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The Role of Molecular Chaperones in the Etiology and Treatment of Psychiatric Diseases in the Elderly

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The Role of Molecular Chaperones in the Etiology and Treatment of Psychiatric Diseases in the Elderly

by

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DEDICATION

I dedicate this dissertation to my wife, Samantha, whose love is the fuel of my creative energy. Second, to my mother, Julia, who has loved me, raised me, sacrificed for me, and brought me to this wonderful country. Also, to my Dad, John, who has always been there for me, even though we were far apart; you have helped me make the most important and best decisions in my life. Also to my grandfather, Dr. John C. O'Leary Sr., you are the best grandfather a person can have, and I would not have had my education without you; you have allowed me to have so many opportunities and taught me so much. And finally to Alejandro, Mark, and CD, my brothers. I love you all with all my heart.

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ABSTRACT

The elderly are at increased risk for developing psychiatric diseases, which include Alzheimer's disease, depression, anxiety and suicide. The probability of multiple disease comorbidity is also increased in the elderly. At the cellular level, the loss of protein homeostasis is often at the root of disease emergence, and thus the scientific community is searching for ways to help maintain this balance. A vast group of proteins that are paramount to balancing and counterbalancing protein levels is the molecular chaperone protein group, which has evolved a tremendous variety of functions in the cell. They aid in protein trafficking, folding, receptor signaling, neurotransmission, vesicle forming and fusion, protein degradation, and apoptosis, among other activities. Despite their best efforts, disease still ensues, but because of their vast number and multiple abilities, it may be possible to modulate these proteins as a way to treat and prevent disease. Chaperones are of particular interest in diseases of aging, because chaperone induction and effectiveness is reduced with age. In addition, many diseases of the elderly are

brought on by aberrant protein accumulation, like Alzheimer’s disease. As a result, the hypothesis of this dissertation is whether the modulation of molecular chaperones changes disease pathology. A molecular chaperone family that is important to protein degradation is the Hsp70 chaperone complex. Hsp70 proteins have specialized function depending on cell type and cellular compartment, but Hsp70 proteins are very important for protein synthesis and degradation. As a result, they are in a position to contribute to the regulation of proteins that become aberrant.

In recent years scientific literature has indicated that compounds that inhibit the enzymatic ATP hydrolysis of these proteins promote tau degradation, which accumulates in Alzheimer’s disease. Alzheimer's disease is the sixth leading cause of death in the U.S., it is a progressive neurodegenerative disease, and is caused by the aberrant accumulation of the amyloid beta and tau proteins. Here, we show that treatment with the Hsp70 inhibitor methylene blue, reduces tau, saves neurons, and restores cognition, in a mouse model of tau accumulation (rTg4510). Cognitive rescue occurred despite a severe tangle load, equal to control treated tau transgenic mice. This study shows that reducing soluble tau can restore cognition, reducing tangles is not necessarily to ameliorate cognition, and saving neurons is not

sufficient to increase cognition if they are burdened with soluble tau. This work shows that methylene blue does not affect the the number of tau tangles in this model, as suggested by *in vitro* data. It also suggests that further work into the development of Hsp70 ATPase inhibitors may find success in alleviating the soluble tau burden found in Alzheimer's disease.

The co-chaperone FKBP5 is also of extreme importance, not because it is essential, but because research has implicated this protein with a host of psychiatric diseases. Single nucleotide polymorphisms in this gene, which increase the levels of FKBP5, interact with averse traumatic events to enhance the likelihood of developing mood and anxiety disorders, including major depressive disorder, post-traumatic stress disorder, bipolar disorder, and suicide. Moreover, we have found that FKBP5 protein levels increase with age in the human brain, increasing the risk for the elderly of developing disease if exposed to traumatic stress. Here, we tested the hypothesis that FKBP5 negatively regulates resilient behavior. We found that FKBP5 levels increase with age in the wild type mouse brain, and that wild type mice display reduced resiliency with age. FKBP5-/- mice, on the other hand, show enhanced resiliency to stress at all ages tested, and are protected from aging-induced despair. At the molecular level,

FKBP5 is a robust inhibitor of the glucocorticoid receptor, which is responsible for the shut-off of the hypothalamic-pituitary-adrenal axis. In addition, excess glucocorticoid levels in the blood is a robust marker of psychiatric disease. Consequently, FKBP5 may be causing disease through enhanced levels of glucocorticoids. FKBP5-/- mice display reduced corticosterone after stress. Moreover, corticosterone production increases with age, and FKBP5-/- mice are protected from this increase. These studies are the first to show that reducing the levels of FKBP5 is a promising therapeutic option for the treatment of mood disorders in the elderly, resiliency naturally declines with age due to FKBP5, corticosterone levels after stress rise due to FKBP5, and that the ablation of this gene increases resiliency and prevents aging-induced despair.

As a whole, these data show that the modulation of chaperone proteins has the potential for developing new therapies for the treatment of psychiatric diseases of the elderly.

CHAPTER ONE:

Introduction

The explosion in medical research, discovery, and access to medicine that occurred in the 20th century, dramatically increased the lifespan of people all over the world. There was considerable growth in the number of individuals reaching the age of 65 in the United States, from 4.1% in 1900 to 12.4% in 2000 (Daccache et al., 2011). Notwithstanding this increase of life expectancy, aging increases susceptibility to psychiatric diseases (Sibille, 2013). And in spite of the growing accumulation of medical knowledge, relatively minuscule advancement has occurred in the development of hypothesis-driven therapeutic approaches. As a result of this, this dissertation is focused on the testing of hypotheses aimed at discovering novel methods to treat psychiatric disorders that affect the elderly.

Alzheimer's Disease

Alzheimer's as a psychiatric disease

Alzheimer's disease (AD), is traditionally considered a neurological disorder. However, it is also officially sanctioned as a psychiatric disorder in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR). From the perspective of the mind, AD is characterized by the loss of learning and episodic memory (Citron, 2010), personality changes (Robins Wahlin and Byrne, 2011), and the loss of general cognitive and intellectual ability. Often, the loss of motivation, anhedonia, and the overlap of symptoms with depression can lead to a misdiagnosis(Landes et al., 2001; Landes et al., 2005). In the later stages AD can produce aphasia (Weintraub et al., 2012), and complete loss of the performance of basic human activities, like eating, dressing, bathing, and even going to the bathroom (Desai and Grossberg, 2001). As of the date of this writing, AD can only be medically diagnosed postmortem, by the paired presence of amyloid beta plaques and neurofibrillary tangles in the brain (Ittner and Gotz, 2011). AD was first recognized by Alois Alzheimer in 1907, and has been researched ever since, marking this year as the 105th year since its discovery (Hippius and Neundorfer, 2003). Notwithstanding, there is currently no cure, preventative method, or disease-modulating agent.

What should we target?

There is some debate in the AD field as to what biological target will yield a cure. As a whole, the answer to the question "what should we target" is "everything." We need to target all the different problems in AD, like amyloid beta, tau, inflammation, blood brain barrier deterioration, etc. Each one is likely to contribute to the extension of life of the patient. Here, however, we have taken the route of exploring the therapeutic options to reduce the tau burden.

Historically there has been an overrepresentation in the scientific community of scientists and clinical trials that searched for therapeutic avenues that reduce amyloid beta (Lee et al., 2007). This was due to the strong evidence that amyloid beta is the initiator of the AD cascade, including mutations that cause early onset (St George-Hyslop et al., 1987), and, recently, the discovery of SNPs in the APP gene that protect against Alzheimer's (Jonsson et al., 2012). However, key discoveries have shown that tau is of equal importance in AD research.

Mutations have been found in the tau gene that are responsible for causing frontotemporal dementia with Parkinsonism linked to chromosome 17. These mutations produce neurodegeneration and neurofibrillary tangles that are very similar to those in AD. Thus, the

logical conjecture arose that tau, like in FTDP-17, may be capable of producing neurodegeneration in AD. Mice transgenic for the human APP gene do not develop tau tangles, but displayed robust cognitive deficits (Spires and Hyman, 2005). The role of tau in AD was subsequently diminished, as it was presumed that tau was not playing as much of a role. A study from Lennart Mucke's lab, on the other hand, showed that when the tau gene is knocked out in mice transgenic for the human APP gene, cognition is improved dramatically (Roberson et al., 2007). The improvement of these mice ensued, despite robust amyloid beta pathology. Additionally, evidence from the human brain also shows that amyloid beta pathology is not the sole source of AD, since there are people with a heavy amyloid burden in the brain who do not develop AD (Crystal et al., 1988).

What kind of tau should we target?

In healthy individuals, the microtubule associated protein tau, referred to herein as tau, is responsible for stabilizing microtubules, which allow for the transport of materials throughout the cell (Brunden et al., 2009). Tau, though, is the main component of the neurofibrillary tangles found in AD (Brunden et al., 2009). Tau tangles are also called tombstone tangles, since they are so insoluble they are often found at the place of death of neurons. Because of this, the hypothesis was

formed that the formation of tau tangles is toxic for neurons and causes them to die. The current scientific thought about tau, though, is shifting due to research in the last decade that suggests otherwise.

In the publication describing the making of the rTg4510 tau transgenic mouse, the authors utilized the model to test if suppression of tau transgene expression would suppress tau pathology, memory loss, and neuron loss (Santacruz et al., 2005). Tau transgene suppression, via doxycycline, alleviated memory and neuron loss, but neurofibrillary tangles continued to form. Thus, separating tau tangle pathology from memory and neuron loss. This was the first indication that excess soluble tau may be the culprit of acute, deadly neurotoxicity, and not the tau tangles themselves. It also showed that a reduction in soluble tau can be therapeutic, despite the increased presence of tau tangles. Previous to this study it was thought that without a reduction in tangle pathology, cognitive improvement was not possible.

The work of Bradley Hyman shows that the emergence of tangles in the rTg4510 mice does not cause the cell to die acutely, as they can monitor the same neuron for up to 5 days (de Calignon et al., 2010). Further examination of tangle bearing neurons in the cortex of 7-8

month old rTg4510 mice showed intact nuclei. Additionally, histological sections of mice with six weeks of suppressed tau transgene expression shows a large number of tangles, suggesting that tau tangles are long lived and relatively stable. These data suggest that tau tangles are not responsible for acute neuronal death experienced in AD, and that tau neurofibrillary tangles are an atypical or later-stage cause of neuron loss. Neurofibrillary tangles correlate with neuronal death, but fall short in number to account for the size of the neuron loss experienced (Gomez-Isla et al., 1997). In support of this notion that soluble tau is the most toxic tau to neurons it was found that mice overexpressing human wild type tau develop memory deficits, despite the absence of neurofibrillary tangle formation in this model.

These data, as a whole, indicate that the depletion of soluble tau could be a successful therapeutic strategy in the treatment of AD. Tau knockout animals are also functionally intact (Ke et al., 2012), although they do develop motor deficits with age. This may be due to changes in development, since the gene is absent at this critical period. The development and testing of conditional tau knockout mice has yet to be determined. However, considering the vast majority of AD patients are older than 65 it is not unreasonable to think that a tau-reducing therapy may be suitable for the treatment of AD.

How should we target soluble tau?

One possibility for tau reduction is the modulation of the degradation of the tau protein (Jinwal et al., 2009; Abisambra et al., 2010; Jinwal et al., 2010). Molecular chaperones offer this possibility because they are responsible, among other things, for the proper degradation of proteins through many of the cells degratory pathways, including proteasomal and autophagic protein degradation.

Molecular Chaperones

What are molecular chaperones?

Molecular chaperones are a family of proteins that are dedicated to maintaining the proper function of proteins in the intercellular milieu (Muchowski and Wacker, 2005). They assist in *de novo* protein folding, and in protein refolding after a cellular stressor has caused protein misfolding to occur (e.g. heat shock) (Barral et al., 2004). Apart from protein folding, molecular chaperones are also necessary in protein transport, targeting, degradation, signal transduction, apoptosis, and vesicle fusion, among other things (Muchowski and Wacker, 2005).

During a cellular stress event all the major families of heat shock proteins get transcribed, including Hsp90, Hsp70, Hsp60, Hsp40, and

Hsp20 families (Lindquist, 1986). However, chaperone proteins cannot always fix the misfolded protein. As a result, the cell has evolved chaperone-mediated targeting of proteins to the degratory pathways. This occurs through the coordinated crosstalk between Hsp70, Hsp90 and representative proteins of the different degradation pathways, like E3 ubiquitin ligases, and lysosome-associated membrane proteins (Ballinger et al., 1999; Massey et al., 2006).

Protein misfolding occurs despite chaperones

It is thought that the induction and function of molecular chaperones decrease with age (Muchowski, 2002; Soti and Csermely, 2002). Moreover, loss of Hsp70 chaperone function, via the overexpression of a dominant negative form, has been shown to produce neurodegeneration in a fruit fly. In addition, there is a reduction in autophagy and proteasomal degradation with age. Accordingly, there may be a reduction in the ability of neurons to handle cellular stressors that require the protein activity of molecular chaperones. This would lead one to the hypothesis that increasing the activity of chaperones would reduce the aggregation of proteins, and the subsequent neurodegeneration this produces, at least for tau.

Molecular chaperone levels or activity?

Despite the fact that chaperones decrease with age, research has shown that chaperone stimulation is not always effective in the management of aberrant tau accumulation (Jinwal et al., 2009). In 2009, the use of a high throughput screening system identified chemical compounds that modulate the ATPase activity of Hsp70. Oppositely of what the literature was suggesting, Hsp70 ATPase activating compounds promoted tau accumulation, and Hsp70 ATPase inhibiting compounds promoted tau degradation in cell models of tauopathy (Jinwal et al., 2009).

The co-chaperone CHIP, C-terminus of Hsc70 interacting protein, is an E3 ligase that works with Hsp70 and Hsp90 to promote tau degradation. CHIP reduces the ATPase activity of Hsp70, and helps ubiquitinate misfolded proteins that are beyond the repair that chaperones can provide (Xu et al., 2002). Congruent with the idea that chaperones help misfolded proteins, CHIP levels are reduced in the rTg4510 tau mouse model (Dickey et al., 2009). Moreover, knockout of CHIP exacerbates tau accumulation (Dickey et al., 2006). However, overexpression of CHIP enhances folding activity, not degratory activity of Hsp70. On the other hand, Hsp72 overexpression was found

to reduce the levels of tau in a cell model (Jinwal et al., 2013), and a mouse model (Petrucelli et al., 2004).

These studies demonstrate that all chaperones are not made equal, and the exact circumstance that may lead to tau degradation has to be worked out.

FKBP5 and Psychiatric Diseases

Mood and anxiety disorders in the elderly

The percentage of people with major depressive disorder and other mood disorders does not increase with age. In fact, approximately 1% of older adults meet the DSM-IV criteria for major depression (Weissman et al., 1988). This is substantially lower than in younger individuals. Generally, mood and anxiety disorders tend to decrease with age, despite the brain having less regenerative power, and a general decline in health (Byers et al., 2010). Nonetheless, 15-25% of the elderly population experience depressive symptoms that interfere with their quality of life (Koenig and Blazer, 1992). Depression is the most common disease in the elderly, according to the American Psychiatric Association. This phenomenon can also be attributed to different factors, like the cohort effect, survivor bias,

sampling methods, and epidemiological and diagnostic issues in the proper diagnosis of disease in the elderly (Byers et al., 2010). There is also a tendency to underreport mood and anxiety disorders in the elderly (Wiener et al., 1997). This is evident in the suicide statistics; suicide rates increase steadily and culminates in old age . The general decline of the body with age gives these individuals reduced ability to recover if extreme trauma is experienced; 71-97% of older adult suicide victims suffer from a psychiatric illness. In terms of population growth, the giant baby boomer generation is quickly coming to a head, as they are beginning to age past 65 years, a critical barrier for the onset of age-related disorders. And although the percentage of ill individuals may drop with age, the number of people with psychiatric disorders aged 65 and older is only going to increase.

FKBP5

FKBP5 is a co-chaperone of the Hsp90 chaperone complex. The Hsp90 machinery is part of all hormone receptors, including androgen, estrogen, mineralocorticoid, and glucocorticoid receptor (GR). The glucocorticoid receptor is of great importance because glucocorticoid resistance is one of the most commonly observed irregularities in mood disorders (Pariante and Miller, 2001). FKBP5 is a potent inhibitor of GR, and is the cause of a naturally existing form of glucocorticoid

receptor insensitivity in several species of new world monkeys (Reynolds et al., 1999; Denny et al., 2000; Scammell et al., 2001; Wochnik et al., 2005; Westberry et al., 2006). In addition, there are single nucleotide polymorphisms in the GR gene, *NR3C1*, that are associated with major depression (van Rossum et al., 2006).

Discovery of SNPs in the FKBP5 gene

GR activity in the hypothalamus and pituitary is responsible for the inhibition of the hypothalamic-pituitary-adrenal (HPA) axis that produces glucocorticoids in response to a stressor (de Kloet et al., 2005), creating a negative feedback loop. Since basal hypercortisolemia is common in depression, Binder et al., 2004, hypothesized that genes that regulate the HPA axis, especially those modulating GR sensitivity, could contribute to the susceptibility of developing depression. They performed a search for SNPs in the *NR3C1, BAG1, STUB1, TEBP, FKBP4, FKBP5, CRH,* and *AVP* genes*, and* found that although none of the SNPs were statistically significant, they found that FKBP5 SNPs significantly associated with a faster response to antidepressant treatment. In particular, they looked at the SNP rs1360780. They genotyped depressed patients at this location in the FKBP5 gene and found an overrepresentation of the TT genotype,

which corresponds to people that inherited a thymidine at this location from both parents, hence "TT." Although a quicker response to an antidepressant treatment is definitely a positive phenotype, these patients also develop a staggering number of depressive episodes throughout their life, as compared to other depressed individuals that have the CT or CC genotypes. The TT individuals also produce excess FKBP5.

After this initial discovery other studies began to emerge looking at SNPs in the FKBP5 gene, and several more have been discovered and found to be overrepresented in people who experience trauma and develop major depressive disorder (Zimmermann et al., 2011), post-traumatic stress disorder (Binder et al., 2008), commit suicide (Roy et al., 2010; Roy et al., 2012), display aggressive behavior (Bevilacqua et al., 2012), and develop bipolar disorder (Willour et al., 2009).

Interestingly, the experience of trauma is highly important for the development of psychiatric disease, as the majority of these require it for the associations with the SNPs to be significant, as was the case for depression. In fact, the mechanism for this has recently been found (Klengel et al., 2012).

Gene x environment in FKBP5

Gene times environment is the emergence of a phenotype based on the combination of a predisposing genotype in an environment that allows the phenotype to express (Meaney, 2010). SNPs that promote smoking behavior, for example, require an environment where an introduction to smoking is possible (Duncan and Keller, 2011). Due to the ability of this interaction to apply a context to the otherwise isolated genome, and its ability to explain natural phenomena, this approach to research has become very popular in the last decade. This applies to FKBP5 and GR, since they are stress reactive proteins. As a result, the combination of stressful environments and genetics may help explain why the same stressor produces disease in some but not others.

Gene x environment works with FKBP5 and GR in the following way. GR produces its effects by the combination of the transactivation and transrepression of ~100 genes (de Kloet et al., 2005). One of the genes that is transactivated is FKBP5 (Scharf et al., 2011). FKBP5 contains several hormone responsive element sequences, which allow it to be activated by hormone receptor complexes, including the progesterone and androgen receptor, in addition to GR (Barik, 2006). As a result, GR activity has a built-in attenuation. In this way, FKBP5

serves as a GR activity rheostat. People who hare homozygous for the T allele of the FKBP5 SNP rs1360780, display greater FKBP5 expression (Binder et al., 2004). This single nucleotide change increases the binding between the RNA polymerase II and the TATA box, yielding higher FKBP5 transcription (Klengel et al., 2012). FKBP5 levels are further increased by stress. This increased level of FKBP5 protein allows for the increased production of glucocorticoids after stress. However, glucocorticoids promote the demethylation of genes, one of which is FKBP5. This, in turn, is a positive feedback loop that begins with an SNP, which is small, and in combination with extended traumatic stress starts a snowball effect that results in a epigenetic change in the FKBP5 gene that may be permanent.

Why is FKBP5 relevant to aging

The risk that FKBP5 poses is even greater with age, because the basal expression levels of FKBP5 increase in the human brain with age (Blair et al., In Press). In fact, they increase age-dependently due to the natural demethylation of the FKBP5 gene (Figure 1.2). As a result of this, elderly people in general, even those without predisposing SNPs, may be at greater risk for developing disease if they are exposed to environments that are extremely traumatic. Elderly individuals who are carriers of the risk alleles are at even greater risk

of developing disease from traumatic stress. As a result, it is extremely important to better understand the role of FKBP5 in the development of mood and anxiety disorders.

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CHAPTER TWO[1]:

Phenothiazine-Mediated Rescue of Cognition in Tau Transgenic Mice Requires Neuroprotection and Reduced Soluble Tau Burden

Abstract

Background

It has traditionally been thought that the pathological accumulation of tau in Alzheimer's disease and other tauopathies facilitates neurodegeneration, which in turn leads to cognitive impairment. However, recent evidence suggests that tau tangles are not the entity responsible for memory loss, rather it is an intermediate tau species that disrupts neuronal function. Thus, efforts to discover

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therapeutics for tauopathies emphasize soluble tau reductions as well as neuroprotection.

Results

Here, we found that neuroprotection alone caused by methylene blue (MB), the parent compound of the anti-tau phenothiaziazine drug, Rember™, was insufficient to rescue cognition in a mouse model of the human tauopathy, progressive supranuclear palsy (PSP) and fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP17): Only when levels of soluble tau protein were concomitantly reduced by a very high concentration of MB, was cognitive improvement observed. Thus, neurodegeneration can be decoupled from tau accumulation, but phenotypic improvement is only possible when soluble tau levels are also reduced.

Conclusions

Neuroprotection alone is not sufficient to rescue tau-induced memory loss in a transgenic mouse model. Development of neuroprotective agents is an area of intense investigation in the tauopathy drug discovery field. This may ultimately be an unsuccessful approach if soluble toxic tau intermediates are not also reduced. Thus, MB and related compounds, despite their pleiotropic nature, may be

the proverbial "magic bullet" because they not only are neuroprotective, but are also able to facilitate soluble tau clearance. Moreover, this shows that neuroprotection is possible without reducing tau levels. This indicates that there is a definitive molecular link between tau and cell death cascades that can be disrupted.

Introduction

The current clinically available options for treating Alzheimer's disease (AD) are limited to acetylcholinesterase inhibitors and NMDA receptor antagonists (Doody, 1999; Reisberg et al., 2003). For tauopathies like PSP and FTDP17, treatment is restricted to supportive therapies. Thus the demand to identify compounds that can remove the microtubule associated protein tau is extremely high.

Modifying tau pathophysiology has been the primary goal of first-generation tau therapeutics. For example, kinase inhibitors (Mazanetz and Fischer, 2007), microtubule stabilizers (Zhang et al., 2005), tau aggregation inhibitors , immunotherapy (Sigurdsson, 2009), and chaperone-based drugs targeting disease-specific tau species (Dickey et al., 2007), have all been proposed based largely on

in vitro data. However, their efficacy for ameliorating cognitive deficits in mouse models of tauopathy have not been tested, primarily due to the fact that few models of tau accumulation are available that develop memory loss. One of the more controversial tau modifying compounds, to recently emerge as a potentially clinically relevant drug, is the phenothiazine methylthionium chloride better known as methylene blue (MB). MB is best known for its function in the laboratory as a redox indicator and as an antiseptic (Oz et al., 2009); however, it, along with other phenothiazine derivatives, have been used extensively in the clinic since the 1950's to treat a number of different conditions, including schizophrenia, mania, anxiety, emesis, cancer, high blood pressure, allergies and even parasitic infections (Mosnaim et al., 2006). These compounds are generally well tolerated and have minimal side effects, including discoloration of urine and ocular vitreous.

More recently MB has been shown to inhibit the aggregation propensity of proteins that can adopt a ß-sheet conformation in vitro (Medina et al.; Taniguchi et al., 2005; Necula et al., 2007), and it was this property that critically linked MB to AD as a possible plaque or tangle buster. However, the propensity of the phenothiazines to

liberally bind to proteins and donate electrons has resulted in a number of other mechanisms being ascribed to them. For example, MB can regulate mitochondrial function (Hassan and Fridovich, 1979; Visarius et al., 1997) and inhibit Hsp70 ATPase activity (Deiana et al., 2009; Jinwal et al., 2009). Interestingly, these pleiotropic mechanisms and clinical applications combined with its relatively innocuous side effects and high bioavailability are what make the phenothiazines such an interesting therapeutic option for tauopathies. Most of the outcomes ascribed to MB could converge to ameliorate symptoms associated with tau accumulation. Here, we sought to determine whether MB could potentially be beneficial as a therapeutic option for tauopathies, based on its pleiotropic anti-tau efficacy, we investigated how its chronic administration might impact the rTg4510 tau transgenic mice. We show that MB is capable of protecting neurons, however, only high dose MB treatment was able to reduce tau and also improve cognition. However, pathology was unaffected. This shows that MB does not reduce tau pathology, but reduces soluble tau levels. Also, it shows that neuroprotection alone is not sufficient to improve behavior, but only when MB levels are sufficiently high to reduce soluble tau levels can memory be improved.

Materials & Methods

Mice

The rTg4510 mice and parental mutant tau and tTA lines were generated and maintained for this study as previously described (Santacruz et al., 2005).

Central administration

A concentration of 1 mM MB in saline was infused by pump into the CA3 of the right hippocampus of rTg4510 mice. Alzet pumps (Model 1004, 100µL, 0.11 µl/hr; Alzet Osmotic Pumps) were filled with 100 µl of 1 mM MB or with saline 0.9% . The pumps were incubated in 0.9% saline at 37°C for 48 hr. Mice were operated in a stereotaxic apparatus (51725D, Stoelting, Wood Dale, IL). A mid-sagittal incision was made to expose the cranium and a small aperture was drilled with a dental tool over the right hippocampus to the following coordinates from bregma: anterior-posterior, -2.7 mm; lateral, -2.5 mm. The osmotic pump was inserted into a subcutaneous pocket on the back of the mouse, leading the catheter to the site of cannula (Brain infusion kit 3, Alzet) placement. The cannula, with a thin layer of cyanoacrylate (Loctite 454, Alzet), was attached to the stereotaxic cannula holder

(Cannula holder 51636, Stoelting) and then lowered 3 mm ventral through the midline aperture. The incision was cleaned with saline and closed with surgical sutures. After surgery, the mice were housed individually. Infusion lasted for 28 days. Six mice were administered with MB and 7 with Saline. The mice were 7 months old at the time of surgery and they were 7 months and three weeks old at the time of water maze testing.

Peripheral administration

Ten age and gender-matched rTg4510 mice and ten wild type littermates were administered MB (Sigma) and saccharine (Acros Organics, Geel, Belgium) in their drinking water, while another ten rTg4510 mice and ten wild type littermates received drinking water with saccharine only. A treatment of 650 mg/day in a 70 kg human equates to 9.3 mg/kg/day. Based on a 4.5 ml of water/day rate of consumption of a 30 g mouse, ad libitum, we estimated mice to receive 9.3 mg/kg/day (0.062 mg/ml). MB or vehicle administration was initiated in 12 week-old rTg4510 and wild type mice using drinking water supplemented with 2mM saccharine. Treatment was maintained for four months and the supplemented water was replaced three times per week. Mice were 6 months old at the time of initial behavioral

assessment and they were 6.5 months old at the time of the water maze analysis. During the course of the study one mouse died of unknown circumstances and drug, tau levels and stereological analyses were not able to measured.

Determination of methylene blue concentrations in the cerebellum

Frozen brain tissue was thawed on ice and homogenized for 2 minutes at a concentration of 100 mg/ml in homogenizing solution (ACN:PBS = 9:1). A 30 µL aliquot of the brain homogenate was added to a 1.5 mL polypropylene microcentrifuge tube and spiked with 30 µL of the internal standard solution (Methylene Violet 3RAX, 250 ng/ml). Analytical grade acetonitrile was then added (90 µL) and the sample was vortexed again for 30 s. Following high speed centrifugation at 13,200 rpm for 10 minutes at 4°C, the supernatant was transferred to a glass vial and subjected to LC-MS analysis. The LC-MS system used for these studies was a Shimadzu (Columbia, MD) series 2010EV instrument equipped with an APCI probe to minimize ion suppression. Quantification was performed using LCMSolution Version 2.05 and a set of external standards.

Open field

The open field is used as a standard test of general activity. Animals are monitored for 15 minutes in a 40 cm square open field with a video tracking software (ANY-Maze, Stoelting, Illinois), under moderate lighting. General activity levels were evaluated by measurements of horizontal and vertical activity.

Rotorod test

This test was performed on an accelerating rotorod apparatus (Ugo Basile, Italy) with a 3cm diameter rod starting at an initial rotation of 4 RPM accelerating to 40 RPM over 5 minutes. Mice were tested for the time spent on the rod during each of four trials with a thirty-minute inter-trial interval. Each trial was completed when the mouse fell off the rod (distance of 12 cm) onto a spring-cushioned lever.

Elevated plus maze.

Anxiety can be assessed through the elevated plus maze (EPM).The EPM consists of two well-lit open arms (35 cm) facing each other and two enclosed arms (30.5 cm) also facing each other. Each

arm is attached to a common center platform (4.5 cm square) and elevated 40 cm off the floor. The mouse is placed in the center platform and allowed to explore for 5 min. Video tracking software measures movement in each section (ANY-Maze, Stoelting, Illinois).

MWM. A circular pool (1.38-m diameter) filled with opaque water at room temperature with an escape platform (15 cm x 15 cm) hidden beneath the water level (3 cm) was used. Each mouse was given four trials per day with an intertribal interval of 1 hour for 6 consecutive days. The time to find the platform (escape latency), the total distance traveled and the swim speed of the animals was recorded. Each animal was given a maximum of 60 seconds to find the platform. During training, if the mice failed to find the platform after 60 seconds, they were placed on the platform for 30 seconds. They were then towel-dried and placed in a cage with a heating pad underneath until dry and returned to their home cage. On day 7 the mice were subject to one probe trial in which the platform was removed and each animal had 60 seconds to search the training pool for the platform.

Brain tissue fractionation and western blot analysis

Brain tissue was homogenized as previously described (Dickey et al., 2006). Measurements of tau levels were performed by western blot analysis.

Immunohistochemistry

Fixed mouse brains were cryoprotected in successive 24 hours incubations of 10%, 20%, and 30% solutions of sucrose and then sectioned as previously described [Gordon, 2002]. Stained sections were imaged using an Olympus BX51 microscope at original 40x, 100x, or 200x final magnification. For quantification, images (original 100x magnification) of cornu ammonis (CA)1, CA3, entorhinal cortex, cortex and dentate gyrus were taken using spatial orientation cues. Quantification of positive staining product was determined using Image-Pro Plus (Media Cybernetics, Silver Springs, MD). Nissl staining was done in 0.05% cresyl violet for 5 minutes followed by differentiation in acidic water until desired color. Tissue was dehydrated through a graded series of ethanol (75%, 95% and 100%). Slides were cleared in Histoclear (xylene substitute) and cover slipped with DPX. Silver stain was performed as previously described (Lewis et al., 2000).

Stereological analysis

Neurons that were stained with cresyl violet were counted in the the cornu ammonis 1 (CA1), the cornu ammonis 2 and 3 (CA2+3), the dentate gyrus (DG), the cerebral cortex (CX) and the striatum (STR) using the optical fractionator method of stereological counting (West et al., 1991) with commercially available stereological software (StereoInvestigator, MBF Bioscience, Williston, VT). A systematic random sampling of sections throughout the left hemibrain were stained as described above and coded to ensure blinding. The regions of interests (ROI) were defined using specific landmarks within the brain to maintain consistency. A grid was placed randomly over the region of interest slated for counting. At regularly predetermined positions of the grid, cells were counted within three-dimensional optical dissectors. Within each dissector, a 1μm guard distance from the top and bottom of the section surface was excluded. Section thickness was measured regularly on all collected sections to estimate the mean section thickness for each animal after tissue processing and averaged 35.24 μm ± 0.46 μm for all sections analyzed. The total number of neurons was calculated using the equation:

$N = Q\text{-} \times 1/ssf \times 1/asf \times 1/hsf$

where N is total neuron number, Q- is the number of neurons counted, ssf is section sampling fraction, asf is the area sampling fraction and hsf is the height sampling fraction. Tissue from one mouse in the MB-treated rTg4510 cohort was unusable for this study. Therefore, n=8 for this group.

Antibodies

Tau antibodies S202/T205 and MC1 were provided by Dr. Peter Davies, Albert Einstein College of Medicine; total tau antibody was purchased from Stantacruz Biotech, Stantacruz, CA. Horseradish peroxidase conjugated secondary antibodies were obtained from Southern Biotech, Birmingham, AL. Glyceraldehyde-3-phosphate dehydrogenase antibody was obtained from Meridian Life Science, Saco, ME.

Statistical analysis

Statistical analysis comparing 2 groups was done using an unpaired, two tailed t-test. Analysis comparing more than 2 groups was done using a one-way analysis of variance (ANOVA) with the Tukey-Kramer post-hoc test. Analysis of three or more groups during

different time points (water maze learning and rotorod) was done using repeated measures 2-way ANOVA with the Bonferroni post-hoc test. Statistical analysis was done in the GraphPad Prism software.

Results & Discussion

Previously, biochemical analyses of hippocampal tissue from tau transgenic mice [rTg4510; (Santacruz et al., 2005)] injected with MB showed reduced tau levels after 24 hours (Jinwal et al., 2009). Hippocampi of wild type mice were then injected with either 1mM or 0.1mM MB [based on a recent study in Zebrafish (van Bebber et al., 2010)] to determine its effective therapeutic window for reducing tau levels. Only 1mM MB reduced tau levels (Figure 2.1 A). Based on these results, 1mM MB (1mM; n=6) or saline (n=7) was administered to the right hippocampi (Smith and Milner, 1981) of 7-month old rTg4510 mice for 1 month using mini-osmotic pump implantation (Figure 2.1 B). The accumulation of soluble mutant human tau in the forebrain of this model causes spatial memory deficits as early as 3 months of age, which precede neuronal loss (Santacruz et al., 2005). Morris water maze (MWM) during the final week of treatment revealed that MB-treated mice showed significant improvements in learning the location

of the escape platform compared to those receiving saline (Figure 2.1 C). Probe trial analysis using target quadrant discrimination, number of platform crossings and search strategy imaging showed significantly improved cognitive recall in MB-treated mice compared to those treated with saline (Figure 2.1 D-F). Biochemical analysis of hippocampal lysates showed that both phosphorylated (S202/T205) and total tau levels were significantly reduced (Figure 2.2 A&B). Thus, despite the focal distribution of osmotic pump administration and the age of the mice, MB was still able to improve cognitive function; an effect that was concomitant with reductions in hippocampal tau levels. Contrastingly, pathology was unaffected (Figure 2.2 C&D). Furthermore, nissl staining was done in order to determine if any damage was done to the hippocampus due to pump implantation. No such damage was found (Figure 2.2 E&F).

Based on this evidence, a new trial was initiated in rTg4510 mice to test the effects of long-term MB administration not only on behavior and tau biochemistry, but also on neuronal survival and tau pathology. Practical limitations with osmotic pump applications required that this study be done using non-invasive peripheral administration. Dose selection for this study was based on two factors: 1) FDA conversion

tables show that a 10 mg/kg dose in a mouse is equivalent to ~1 mg/kg in humans, which is within the range of current MB clinical applications (FDA, 2005; Walter-Sack et al., 2009), and 2) pharmacokinetic analyses showed that MB could concentrate in the brain 500-fold, making the effective concentration (>100μM) possible (Figure 2.3 A&B).

Thus, two groups (n=10) of 3 month-old rTg4510 mice and two groups of 10 wild type littermates received either ~10 mg/kg (165μM; 5x maximum recommended dose) of MB via drinking water supplemented with 2mM saccharine or saccharine water alone (Figure 2.4 A). Following 12 weeks of treatment, behavioral assessment showed no overt alterations in motor coordination or task acquisition (See Appendix A: Figure A1). MWM was then used to assess cognitive function. Probe trial analysis and search strategy imaging showed that MB, but not saccharine, prevented the significant progressive impairment in target quadrant discrimination that is a hallmark of the rTg4510 phenotype (Figure 2.4 B&C). Furthermore, wild type littermates showed normal spatial memory recall irrespective of MB treatment (for visible trial and swim speed see Figure A3). Biochemical analyses of half-brain homogenates (excluding cerebellum) showed a

reduction in soluble tau levels in some mice, but not others (Fig. 5A,B). Histochemical analyses revealed no change in tau pathology in any mice (Figure 2.5 C&D).

Given the variability in behavioral performance as well as reductions in tau levels, we hypothesized that the MB concentration in the brain may have also varied due to the ad libitum administration strategy. To test this idea, brain concentrations of MB were assessed using LC-MS analysis of the cerebellar tissue from these mice. Indeed, MB concentration was positively correlated with MWM performance ($p<0.05$) and was inversely correlated with soluble tau levels ($p<0.05$) (Figure 2.6 A&B). The differences in brain MB concentration between mice could not be attributed to body weight or gender (See Appendix A: Figure A2). Moreover, mice with >470μM MB brain concentration accounted for a preponderance of the effects on memory function and tau reductions, consistent with our previous results showing that very high concentrations of MB were required for anti-tau efficacy (Figure 2.1 A). Surprisingly, stereological assessment of five different brain regions from these mice showed that MB treatment significantly delayed neurodegeneration by ~30% in all forebrain regions of all rTg4510 mice (Figure 2.6 C), but neuronal number failed to correlate

with memory performance or soluble tau levels (Figure 2.6 D, and data not shown).

Post-hoc analysis of the behavioral performances of rTg4510 mice with brain MB concentrations above or below ~470μM (High [MB] or Low [MB], respectively), elucidated that the High [MB] cohort performed equivalent to wild type mice, while the Low [MB] cohort was significantly impaired (Figure 2.7 A). We again used the camera-tracking software to map the areas of the pool most traversed by each cohort (Figure 2.7 B). High [MB] rTg4510 mice were predominantly found in the target quadrant, while the Low [MB] cohort displayed an unguided search strategy. Furthermore, we wanted to see if MB treatment in the High [MB] mice had an effect on motor learning. The mice were subjected to the rotorod task for two days and the latency to fall onto a spring-cushioned lever was measured. We found that motor learning from day 1 to day 2 of MB treated rTg4510 was significantly improved, dissimilar to the saccharine treated rTg4510 analogs (Figure 2.7 C).

Conclusion

In conclusion, these findings are consistent with recent evidence showing that reducing tangle burden does not beget functional recover(de Calignon et al.; Santacruz et al., 2005; Spires-Jones et al., 2008); however, the unexpected result that neuroprotection is insufficient to preserve cognitive function suggests that preventing neuronal loss may not be enough to alter cognitive deficits in tauopathies either. In fact, it was only when soluble tau levels are reduced in the brain that functional recovery was observed. Moreover, these data show that there is a link between tau and cell death signaling cascades that can be altered with therapeutics since neurodegeneration can be decoupled from tau accumulation with MB.

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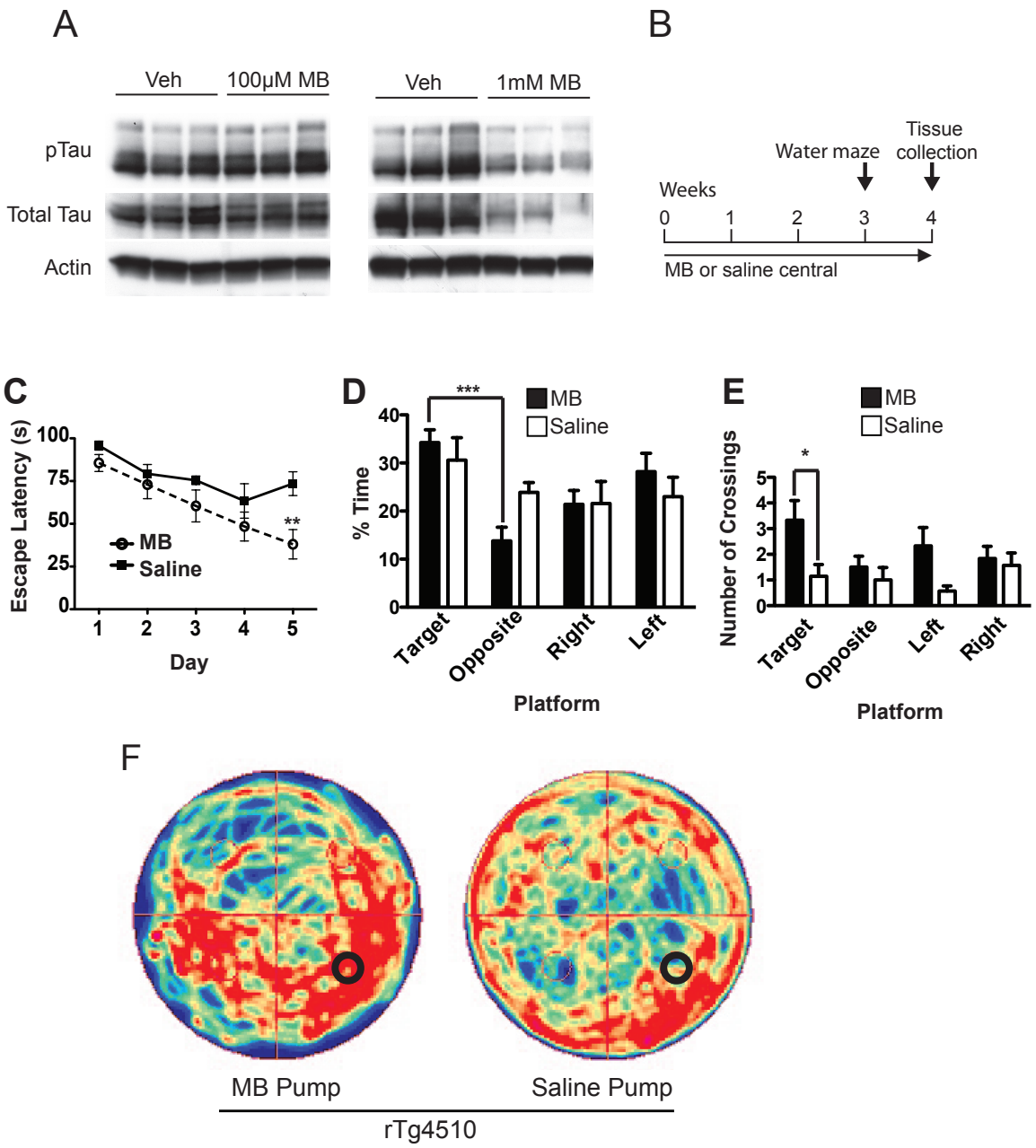
A
Veh
100μM MB
Veh
1mM MB
pTau
Total Tau
Actin
B
Water maze
Tissue collection
Weeks
0
1
2
3
4
MB or saline central
C
Escape Latency (s)
100
75
50
25
0
MB
Saline
**
1
2
3
4
5
Day
D
***
MB
Saline
% Time
40
30
20
10
0
Target
Opposite
Right
Left
Platform
E
MB
Saline
*
Number of Crossings
5
4
3
2
1
0
Target
Opposite
Left
Right
Platform
F
MB Pump
Saline Pump
rTg4510

Figure 2.1

Figure 2.1. Direct hippocampal infusion of methylene blue by mini-osmotic pump reverses spatial navigation related learning and memory deficits by reducing tau levels in rTg4510 mice. (A) Four groups of wild type mice (n=3) were injected with 100 μM MB, 1 mM MB, or saline into the hippocampus for 24 hours. Western blot from hippocampal lysates show reductions in tau at 1 mM MB in phospho- and total tau. (B) Experimental design of central administration of MB (MB central n=6, saline central n=7). (C) Infusion of MB increased ability of rTg4510 mice to learn the location of the hidden platform in the MWM, **p<0.01). (D) Probe trial of Morris water maze shows that MB treated rTg4510 mice were able to recognize the target from the opposite quadrant, unlike saline treated rTg4510 mice, ($F(3,20)=8.202$, $p=0.0009$), ***p<0.001. (E) Number of crossing across the area where the hidden platform was located during training. ($F(7, 44) = 2.757$, $p=0.0183$), p<0.05. (F) Search strategy imaging using a camera tracking system shows that MB treated rTg4510 mice have a more focused strategy than saline treated rTg4510 mice.

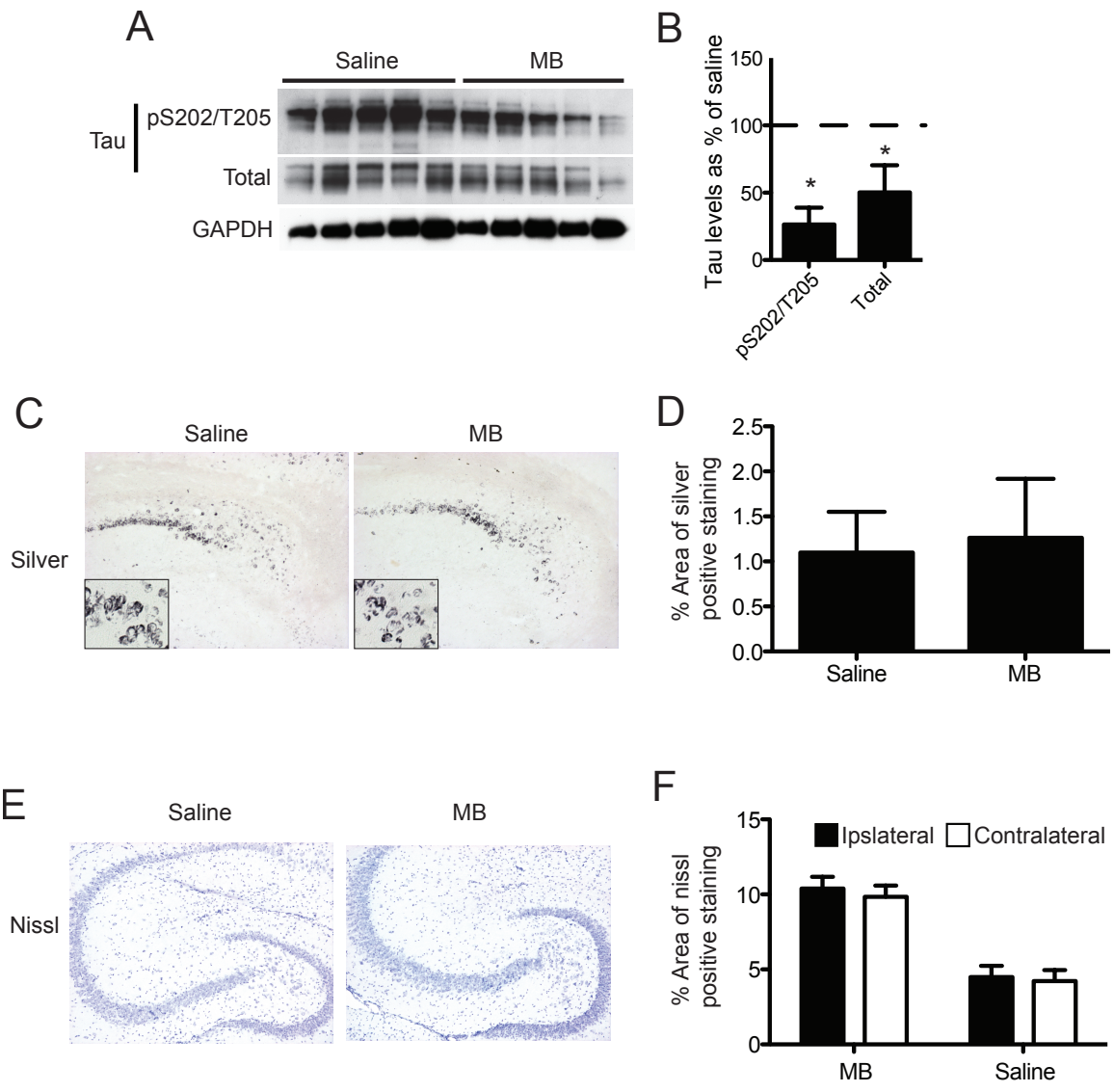
A
Saline
MB
Tau
pS202/T205
Total
GAPDH
B
Tau levels as % of saline
150
100
50
0
*
*
pS202/T205
Total
C
Saline
MB
Silver
D
% Area of silver positive staining
2.5
2.0
1.5
1.0
0.5
0.0
Saline
MB
E
Saline
MB
Nissl
F
% Area of nissl positive staining
15
10
5
0
Ipslateral
Contralateral
MB
Saline

Figure 2.2

Figure 2.2. MB treatment reduces tau levels, but does not affect pathology in mice with pre-existing tangle formation and neurodegeneration. (A) Western blot analysis of tau protein levels from hippocampal lysates. (B) Quantitation of the optical density as a percentage of saline treated rTg4510 mice shows a significant difference in pS202/T205 and total tau levels, *$p<0.05$. (C) Representative images of the Gallyas silver stain. (D) Percentage of area of silver positive staining shows no statistical difference between the means ($n=2$ per group). (E) Representative images of the staining of neuronal nissl substance in the ipsilateral side of drug infusion (right hippocampus). (F) Quantitation of the percent area positive for nissl staining of ipsilateral versus the contralateral brain side of each group of mice shows that there is no detectable neuron loss due to pump implantation.

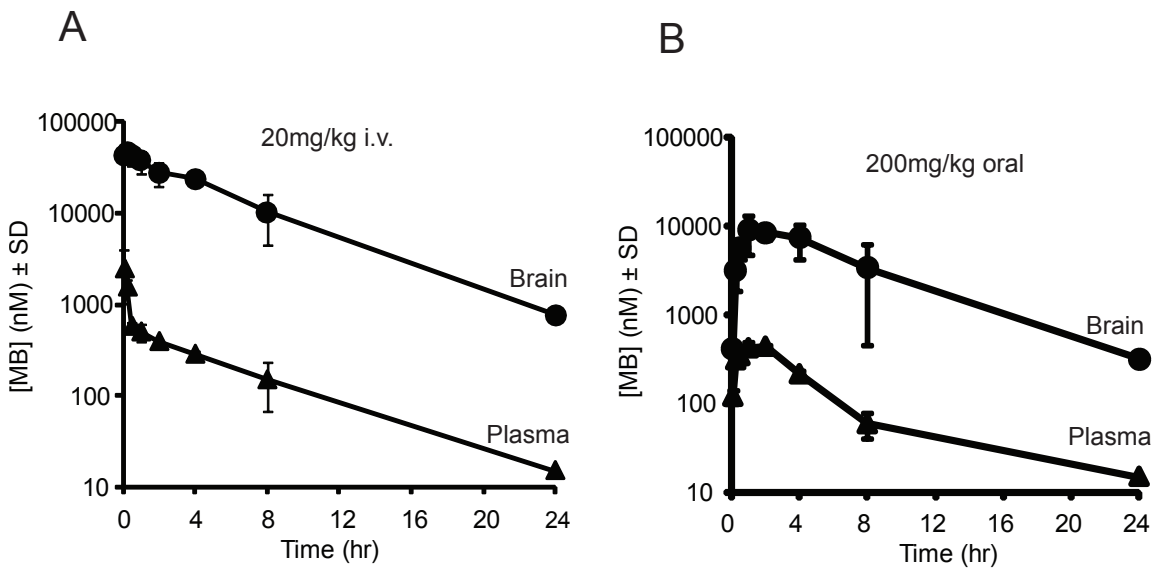
A
B
20mg/kg i.v.
200mg/kg oral
Brain
Plasma
[MB] (nM) ± SD
Time (hr)
100000
10000
1000
100
10
0
4
8
12
16
20
24

Figure 2.3

Figure 2.3. Pharmacokinetic analysis of MB following peripheral administration. (A) A single intravenous (I.V.) administration of 20 mg/kg MB was given to wild type mice at different time points (n=3 per time point), and cerebellar and plasma concentrations were measured through LC-MS. (B) A single 200 mg/kg oral gavage bolus of MB was given to wild type mice at different time points (n=3 per time point) and also measured by LC-MS. Cerebellar brain concentrations of MB are higher than plasma.

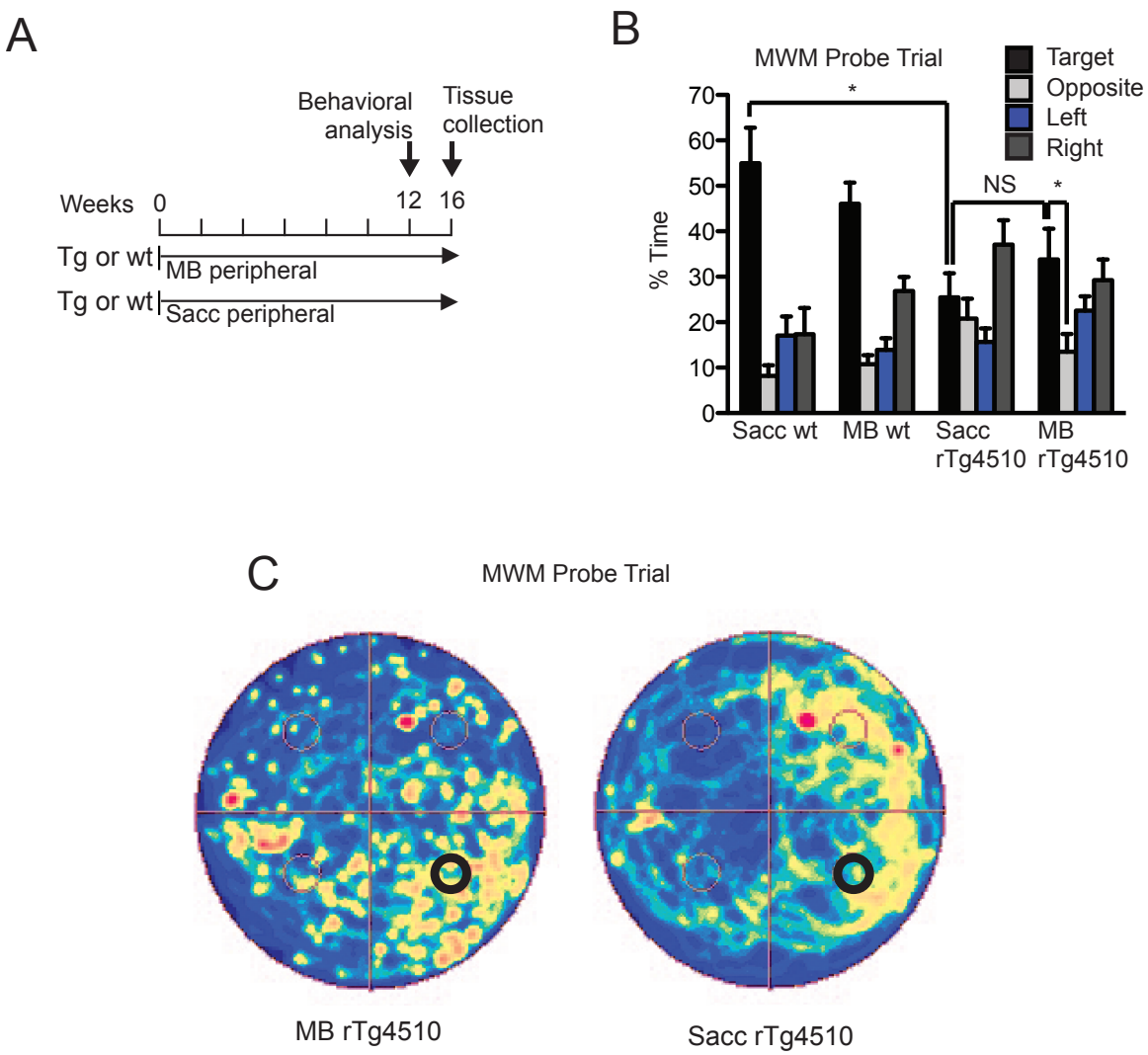
A
Behavioral analysis
Tissue collection
Weeks 0
12
16
Tg or wt
MB peripheral
Tg or wt
Sacc peripheral
B
MWM Probe Trial
Target
Opposite
Left
Right
*
NS
*
% Time
70
60
50
40
30
20
10
0
Sacc wt
MB wt
Sacc rTg4510
MB rTg4510
C
MWM Probe Trial
MB rTg4510
Sacc rTg4510

Figure 2.4

Figure 2.4. Chronic treatment with methylene blue has moderate effect in behavior. (A) Experimental design, n=10. (B) Percent of time spent in each quadrant during the probe trial of MWM ($F(15,144) = 8.781$, $p<0.0001$). MB rTg4510 recognize target versus opposite quadrant ($F(3,36) = 3.38$, $p = 0.0286$) (* $p<0.05$,). (C) Search strategy imaging using a camera tracking system.

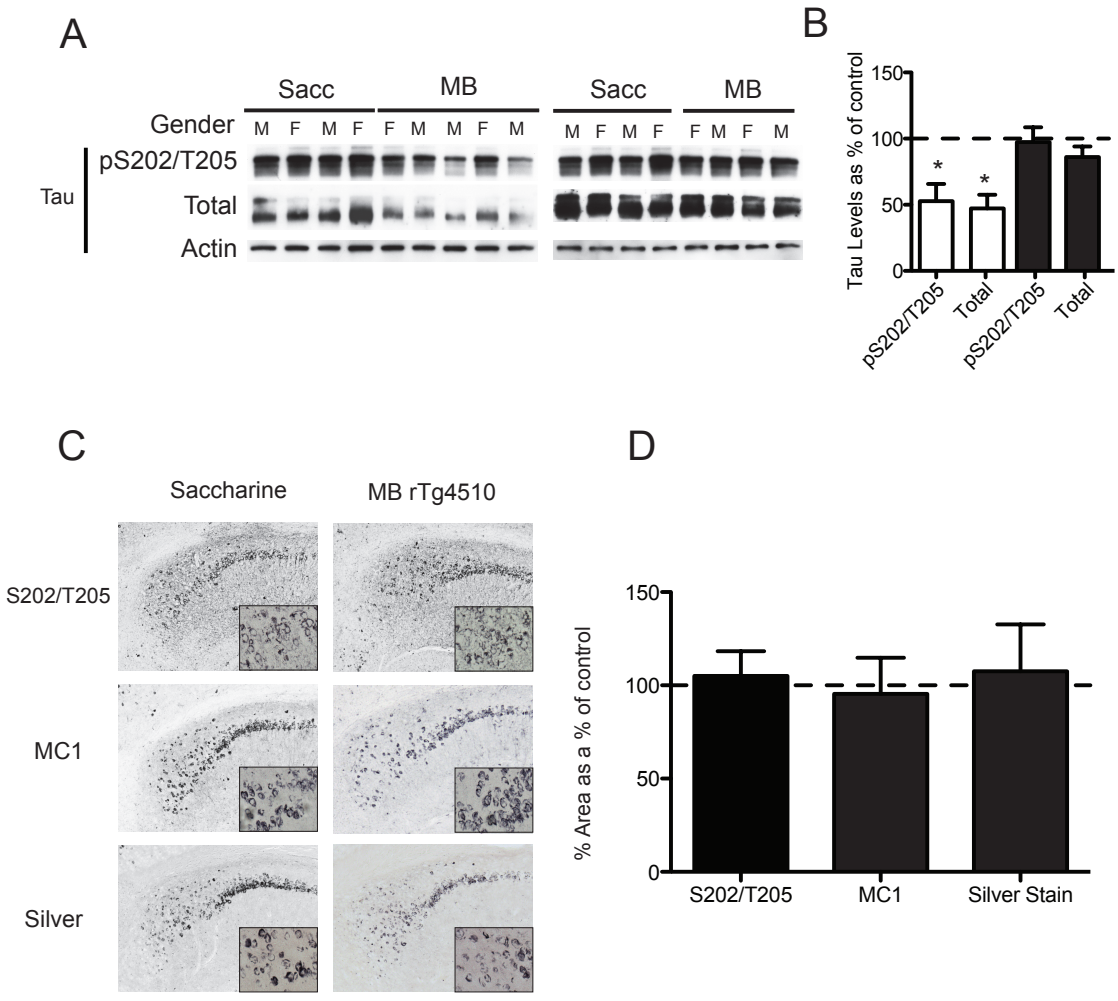
A
Sacc
MB
Sacc
MB
Gender
M F M F F M M F M M F M F F M F M M
Tau
pS202/T205
Total
Actin
B
Tau Levels as % of control
150
100
50
0
*
*
pS202/T205
Total
pS202/T205
Total
C
Saccharine
MB rTg4510
S202/T205
MC1
Silver
D
% Area as a % of control
150
100
50
0
S202/T205
MC1
Silver Stain

Figure 2.5

Figure 2.5. Chronic dosing of methylene blue leads to reductions in soluble tau but pathology is unaffected. (A) Half-brain lysates (no cerebellum) were analyzed for tau protein levels by Western blot. (B) Optical density of tau levels is shown as a percentage of control (saccharine treated rTg4510), (* $p<0.05$). (C) Representative images of immunoreactivity to a phosphorylated tau epitope (pS202/T205), the MC1 epitope (early tangles), and reactivity to the gallyas silver stain (late tangles) are shown. (D) Quantitation of the percent area of positive staining shows no statistical significance between the means of all stains.

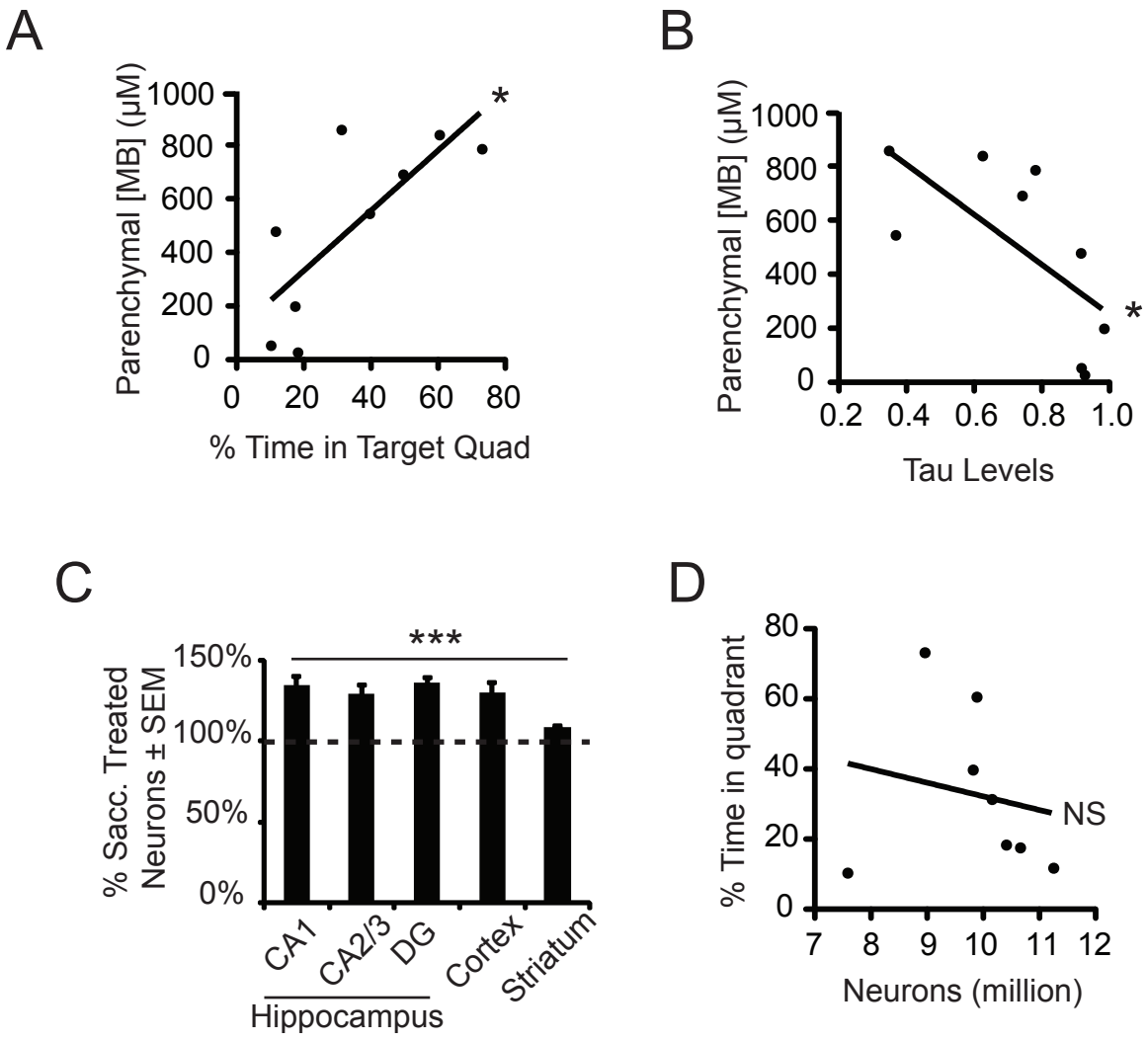
A
Parenchymal [MB] (µM)
1000
800
600
400
200
0
0
20
40
60
80
% Time in Target Quad
*
B
Parenchymal [MB] (µM)
1000
800
600
400
200
0
0.2
0.4
0.6
0.8
1.0
Tau Levels
*
C
% Sacc. Treated Neurons ± SEM
150%
100%
50%
0%
***
CA1
CA2/3
DG
Cortex
Striatum
Hippocampus
D
% Time in quadrant
80
60
40
20
0
7
8
9
10
11
12
Neurons (million)
NS

Figure 2.6

Figure 2.6. Chronic dosing of methylene blue enhances neuronal survival. (A) Parenchymal drug concentrations correlated significantly with memory retention in the probe trial of MWM $p=0.016$, Pearson $r=0.766$, $r^2=0.587$. (B) Tau levels inversely correlate significantly with parenchymal drug concentration, $p<0.05$, Pearson $r=-0.6724$, $r^2=0.452$. (C) Number of neurons counted in five regions as a percentage of sacc treated rTg4510 (MB treated rTg4510 n=8, sacc treated rTg4510 n=10) (DG stands for dentate gyrus, CX stands for cortex and STR stands for striatum). (D) Number of neurons does not correlate significantly with time spent in the target quadrant.

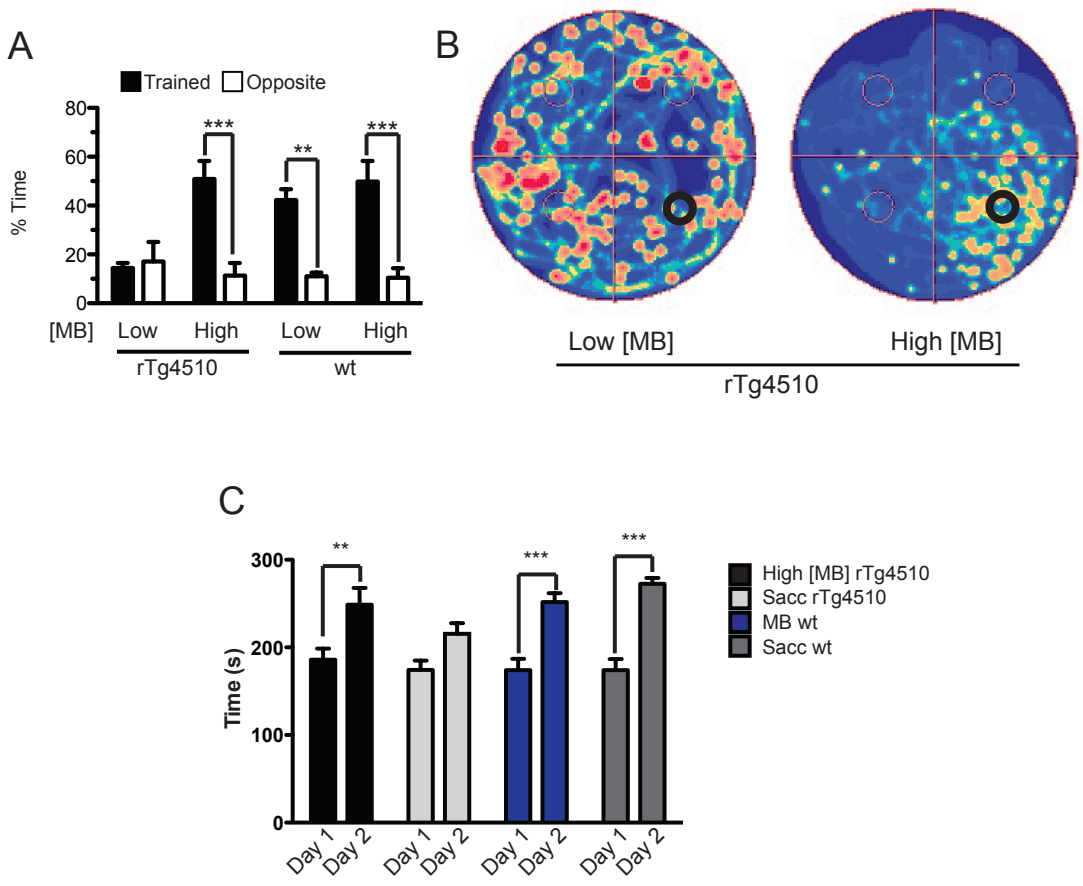
A
Trained
Opposite
% Time
80
60
40
20
0
***
**
***
[MB]
Low
High
Low
High
rTg4510
wt
B
Low [MB]
High [MB]
rTg4510
C
Time (s)
300
200
100
0
**
***
***
Day 1
Day 2
Day 1
Day 2
Day 1
Day 2
Day 1
Day 2
High [MB] rTg4510
Sacc rTg4510
MB wt
Sacc wt

Figure 2.7

Figure 2.7. High concentrations of MB repair spatial and motor memory deficits. (A) Post-hoc analyses reveal that High [MB] rTg4510 mice (n=5) perform similar to wild-type MB treated mice, while Low [MB] rTg4510 mice (n=4) did not display memory retention. Wild type mice performed similarly regardless of MB ($F(7,30) = 10.64$, $p<0.0001$) (**p<0.01, ***p<0.001). (B) Camera tracking software imaging of MB treated rTg4510 mice divided by low and high parenchymal MB concentrations. (C) Motor learning was assessed by comparing the average latency to fall from the rotorod apparatus of day 1 with the average of day 2. High [MB] rTg4510 mice (n=5) learn better than saccharine treated rTg4510 (n=10). Wild Type groups, n=10 (**p<0.01, ***p<0.001).

CHAPTER THREE[2]:

A New Anti-Depressive Strategy for the Elderly: Ablation of FKBP5/FKBP51

Abstract

The gene FKBP5 codes for FKBP51, a co-chaperone protein of the Hsp90 complex that increases with age. Through its association with Hsp90, FKBP51 regulates the glucocorticoid receptor (GR). Single nucleotide polymorphisms (SNPs) in the FKBP5 gene associate with increased recurrence of depressive episodes, increased susceptibility to post-traumatic stress disorder, bipolar disorder, attempt of suicide, and major depressive disorder in HIV patients. Variation in one of these SNPs correlates with increased levels of FKBP51. FKBP51 is also

[2]This work was previously published in the journal *PLoS One* (O'Leary III, John C., et al. "A new anti-depressive strategy for the elderly: ablation of FKBP5/FKBP51." PloS one 6.9 (2011): e24840), and is used here with permission of the publisher. The permissions are available in Appendix F.

increased in HIV patients. Moreover, increases in FKBP51 in the amygdala produce an anxiety phenotype in mice. Therefore, we tested the behavioral consequences of FKBP5 deletion in aged mice. Similar to that of naïve animals treated with classical antidepressants FKBP5−/− mice showed antidepressant behavior without affecting cognition and other basic motor functions. Reduced corticosterone levels following stress accompanied these observed effects on depression. Age-dependent anxiety was also modulated by FKBP5 deletion. Therefore, drug discovery efforts focused on depleting FKBP51 levels may yield novel antidepressant therapies.

Introduction

Genes regulating the hypothalamus-pituitary-adrenal (HPA) axis are associated with susceptibility to depression as well as antidepressant efficacy (Binder et al., 2004; Liu et al., 2006; van Rossum et al., 2006). The HPA axis has a well-characterized role as a regulator of the neuroendocrine stress response (Lupien et al., 2009). Its activation leads to the production of glucocorticoids in the adrenal axis, of which the major constituent in humans is cortisol and in rodents is corticosterone. Over the past decade, genome wide association studies for single nucleotide polymorphisms (SNPs)

revealed significant associations between susceptibility to depressive episodes and variants in both the NR3C1, that encodes the glucocorticoid receptor (GR), and FKBP5, that encodes a GR binding protein thought to attenuate GR activity (Lahti et al.; Binder et al., 2004). While most studies have focused on the variants in GR because of its role as a transcriptional regulator (Derijk et al., 2008), the involvement of FKBP5 and its gene product, FKBP51, have received little attention. This is largely due to uncertainty about how to approach this relatively unknown protein. In fact, it remains to be proven whether FKBP51 is a valid therapeutic target for treating depression, despite its clear genetic link.

Since the initial discovery of the association between FKBP5 SNPs and depression, other psychiatric disorders have been found to be associated with FKBP5 SNPs including PTSD (Xie et al., 2010), bipolar disorder (Willour et al., 2009), anxiety (Binder, 2009), peritraumatic dissociation (Koenen et al., 2005), and major depression in HIV patients (Tatro et al., 2010). The TT variant of the rs1360780 SNP was associated with both an increased incidence of depressive episodes throughout a carrier's lifetime, and increased sensitivity to common neurotransmitter-based anti-depressants (Binder et al., 2004). Interestingly, individuals with the rs1360780 TT SNP had

significantly higher FKBP51 protein levels in their lymphocytes. FKBP51 levels are also elevated in patients with HIV infection, perhaps playing a role in the depression that commonly occurs with chronic highly active antiretroviral therapies (HAART)(Tatro et al., 2009). Recently, stress was shown to induce neuropsin activity in the amygdala, inducing anxiety in mice through an FKBP51-dependent mechanism (Attwood et al., 2011). How FKBP51 directly modulates GR has been investigated in vitro. In these systems, increases in FKBP51 levels decreased GR activation by lowering GR affinity for glucocorticoid (Binder, 2009). In new world monkeys FKBP51 has been shown to be the cause of glucocorticoid resistance, but this has yet to be shown using a genetic in vivo model (Denny et al., 2005; Westberry et al., 2006).

While the causes of major depressive disorders are unknown, there is an emerging genetic diathesis for its occurrence within genes regulating the HPA axis; however few animal models have been developed or utilized for aetiologic validation studies. Genetic variation in FKBP51 appears to be one factor that facilitates liability to anxiety and mood disorders. Thus, the goal of this study was to determine whether decreasing FKBP51 expression could make mice less susceptible to inducible "depression-like" states through a

corticosterone-dependent mechanism in vivo in well established models with high predictive value (Frazer and Morilak, 2005). Indeed, aged FKBP5-deficient mice were resistant to stress-induced depressive-like behavior. Moreover, despite robust hippocampal and forebrain expression patterns, deletion of FKBP5 did not result in cognitive impairment or other behavioral abnormalities. Circulating levels of corticosterone in the same FKBP5-/- mice were also reduced after stress, confirming the proposed mechanism previously described (Binder, 2009). These data suggest that not only is FKBP51 a valid therapeutic target, but targeting this protein may also have minimal consequences for other behavioral characteristics.

Materials & Methods

Generation of FKBP5-/- mice

By PCR screening the 129SvJ mouse BAC library (Genome Systems, St. Louis, MO), bacterial artificial chromosome (BAC) clones that contained genomic regions for FKBP5 were isolated. Restriction fragments were subcloned into pBluescript (pBS; Stratagene, La Jolla, CA) or pZero (Invitrogen, Carlsbad, CA) cloning vectors. The PCR products were amplified from the BAC clones and were then used to construct a targeting vector in the pPGKneo vector (a generous gift of

James Lee, Mayo Clinic Scottsdale). The targeting vector contained a beta-galactosidase/neomycin cassette flanked by regions homologous to the FKBP5 gene and was recombined to remove all of exon 2, which is the first coding exon. ES cells were isolated from the 129SvJ mouse and cultured in Knockout DMEM media (Invitrogen) supplemented with 10% FBS, penicillin/streptomycin, essential amino acids, and ESGRO (103 U/ml; Chemicon, Temecula, CA) with irradiated embryonic fibroblast feeder cells. The ES cells were then electroporated at 0.2 kV, 950μF (Gene Pulser II; Bio-Rad, Hercules, CA) with linearized targeting vectors and selected with G418. DANN from G418-resistant clones was isolated for Southern blot analysis. A DNA probe was used to distinguish PstI restriction fragments from wild-type (~7.5 kb) and mutant (~10 kb) alleles. Appropriate homologous recombination in ES cell clones was confirmed by PCR using primers complementary to sequences within the neomycin cassette and to 3’ FKBP5 sequences downstream from the recombination site. ES cell clones containing a mutant FKBP5 allele were injected into C57BL/6 blastocysts and implanted into pseudopregnant 129SvJ females. Chimeric offspring were identified by coat patterns and mated to C57BL/6 mice to obtain germline transmission of the mutant allele. For colony maintenance mice were crossed from C57BL6 onto Swiss-Webster for purposes of

fecundity and genetic diversity to be more representative of a human population.

Brain tissue fractionation and western blot analysis

Brain tissue fractionation and western blot analysis were done as previously described (Dickey et al., 2006).

PCR. mRNA was isolated and purified from the brain of four wild type and four FKBP5-/- mice using RNAeasy kit (QIAGEN, Valencia, CA). cDNA was synthesized from isolated mRNA by reverse transcription using Super Script III First-Strand cDNA Sythesis Kit (Invitrogen, Carlsbad, CA) from 50ng of isolated mRNA. PCR was performed with synthesized cDNA and FKBP5 specific primers to confirm presence or absence of FKBP5 gene.

Antibodies

Horseradish peroxidase conjugated secondary antibodies (Southern Biotech, Birmingham, AL), Glyceraldehyde-3-phosphate dehydrogenase antibody (Meridian Life Science, Saco, ME), Anti-FKBP51 was provided by Drs. David F. Smith and Marc Cox (Mayo Clinic, Scottsdale, AZ).

Immunohistochemistry

Fixed mouse brains were processed for sectioning as previously described (Gordon et al., 2002). β-galactosidase staining was performed using the in situ β-gal staining kit (Stratagene, La Jolla, CA).

Behavior

N=9 unless otherwise noted. Video tracking software was used in several tests (ANY-Maze, Stoelting, Illinois).

Open Field. Animals were monitored for 15 min in an open field with video tracing software.

Rotorod test

Testing started at an initial rotation of 4 rpm accelerating to 40 rpm over 5 min. Mice were tested for 4 trials per day, for 2 consecutive days with a 30-min intertrial interval. Latency to fall from the rod onto a spring-cushioned lever was measured.

Morris water maze (MWM). Mice were trained to locate an escape platform hidden beneath the water (3 centimeter). Each mouse was given 4 trials per day with an intertrial interval of 1 hour for 6 consecutive days. Each animal was given 60 seconds to find the platform. Afterwards the mice were placed on the platform for 30 s. On

day 7, mice were subjected to a trial in which the platform was removed, and had 60 s to search for it.

Associative fear conditioning

Two mild foot shocks (0.5 milliamps) were paired with an auditory conditioned stimulus (CS, white noise, 70 decibels) within a novel environment. The CS was given for 30 s before each foot shock (2 s). Twenty-four hours later, the mice were placed in the chamber and monitored for freezing for 3 min (no shocks or CS). Immediately after the test, mice were placed into a novel context for 3 min without CS and then exposed to the CS for 3 min (cued).

Prepulse Inhibition (PPI). Mice were placed in a restrainer (Panlab, Barcelona Spain) and placed inside a sound attenuation chamber. The test consisted of 7 trial types in pseudorandom order: 1) 40 ms, 120 decibels sound burst (startle); 2-6) 5 different acoustic prepulses 100 ms in length, a 20 ms duration at 74, 78, 82, 86, and 90 dB; 7) no stimulus for baseline measurement . The intertrial interval was 15 s. The startle response peak was measured within a second after the stimulus.

Elevated plus maze (EPM)

EPM consisted of 2 open arms facing each other and two enclosed arms also facing each other. Each arm is attached to the center platform and elevated 40 centimeters off the floor. The mouse was placed on the platform and allowed to explore for 5 min. Video tracking software measured movement.

Porsolt forced swim test (FST)

Each mouse was placed in a 45 centimeters high and 20 centimeters diameter clear Plexiglas cylinder filled with room temperature water to a depth of 12 centimeters for 6 min. Amount of time spent immobile was recorded.

Tail suspension test (TST)

Mice were suspended from their tail for 6 min. Amount of time spent immobile was recorded.

Novel object

Mice were placed in an area with two objects similar in scale to the mouse. Each animal was given 3 acclimation trials of 5 min with a 5-min intertrial interval. Then one acclimated object was replaced with

a novel object. Animals were given a 5-min exploratory trial monitored by video recording.

Y-maze

Animals were started at the center of the Y and allowed to explore for 8 min. Each session was video-monitored. The number of arm entries was recorded. The percent of spontaneous alternation was calculated as the number of triads containing entries into all three arms divided by the maximum possible of alternations (total number of entries minus 2).

Corticosterone assay, blood collection and stress paradigm

The levels of corticosterone were measured using an ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA). Blood from mice was collected in the morning one hour after the light cycle began and 30 min after a 10-min tube restraint using the submandibular vein puncture method.

Statistics

The student's t-test was used to compare 2 groups. The paired t-test was used to compare paired observations within 2 groups. The 2-way RMANOVA was used to compare the interaction between two

dependent variables and an independent variable. All error bars represent standard error of the mean.

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Results

FKBP5-/- mice were used to determine the effect of gene deletion on behavior. The mice contain a β-galactosidase reporter cassette, which expresses wherever the FKBP5 gene is normally expressed. To confirm gene knockout and establish cerebral distribution of FKBP51, tissue from 5.5 and 20-month-old FKBP5-/- mice was stained using an X-gal kit that produces a blue product when β-galactosidase is present. An age-dependent increase in β-gal expression was observed particularly in the upper cortical layers of the forebrain (Figure 3.1 A). This was consistent with the age-dependent increase in FKBP51 expression that had been previously reported in normal mice (Jinwal et al., 2010). To confirm the absence of FKBP51 protein, whole brain homogenates were analyzed by immunoblot and probed using an FKBP51 antibody. No detectable FKBP51 was observed (Figure 3.1 B). To verify that FKBP51 mRNA was absent, oligo d(T) RT-

PCR was performed using reverse transcriptase to produce cDNA. No FKBP5 PCR product was detected in the FKBP5-/- mice, confirming that no FKBP51 mRNA was present (Figure 3.1 C).

FKBP5-/- mice aged 17-20 months were submitted to two behavioral models of depressive-like activity; the classical forced swim test (FST) and the tail suspension test (TST) (Porsolt et al., 1977; Steru et al., 1985). Results from these tests are based on the total time spent immobile over a 6-minute period, interpreted as despair. These tests are typical for assessing antidepressant efficacy (Steru et al., 1985). Aged FKBP5-/- mice displayed a shorter immobility time than their wild type counterparts (Figure 3.1 D&E). The weight, activity, and physical fitness of the mice were evaluated to account for possible confounding factors, but these variables did not contribute to the effect (Figure 3.1 F-H).

Stress causes increased levels of cortisol in humans and depressed patients have higher-than-normal levels of cortisol in the blood (Burke et al., 2005). The dexamethasone-corticotropin releasing hormone (DEX-CRH) test, a commonly used tool to detect HPA system changes, has been used to link depressive behavior with glucocorticoid receptor (GR) insensitivity (Holsboer et al., 1987). To determine if FKBP5 deletion was altering glucocorticoid levels, circulating

corticosterone levels were assessed in the same aged cohort of FKBP5-/- mice that were used for behavioral analyses. Early morning blood draws were collected from both FKBP5-/- and wild type littermates before and 30 minutes after being placed in a restrainer for 10 minutes. Levels of basal corticosterone were predictably low, consistent with previous reports showing low levels of corticosterone production in the earliest part of the murine diurnal cycle (Kakihana and Moore, 1976)(Figure 3.1 I). After stress corticosterone levels rose in both wild type and FKBP5-/- mice, but this induction was attenuated in the FKBP5-/- mice. These findings suggest that the lack of FKBP51 permits unrestrained GR transcription activity, which decreases HPA-axis activity and corticosterone levels, improving resilience to depressive-like behavior.

However, untoward consequences of FKBP51 ablation were a distinct possibility given the ubiquitous expression of FKBP51 throughout the brain. Given the high levels of expression of FKBP5 in the hippocampus (Figure 3.1 A) memory formation was assessed. Therefore, short-term memory in the FKBP5-/- mice was tested with the use of the novel object recognition and Y-maze paradigms. Both tests are performed in a short period of time to capture the working memory ability of the mice. Neither test showed statistically significant

differences between FKBP5-/- and wild type littermates (Figure 3.2 A&B). Long-term spatial memory function was then tested using the Morris water maze (MWM). Memory retention was tested 24 hours after the training was completed. The training phase of the MWM displayed that wild type and FKBP5-/- mice were equally capable of learning the location of a hidden platform (Figure 2C). Moreover wild type and FKBP5-/- mice displayed an equivalent capacity to locate the hidden platform 24 hours after training (Figure 2D-2E).

The amygdala controls emotional learning in the brain and FKBP5 expression can be upregulated by GR activation (Scharf et al., 2011). As a consequence, function of the amygdala could be affected by FKBP5 deletion. To test the impact of FKBP5-/- on emotionally derived memory function, a fear-conditioning paradigm was employed. In this test, a tone played for 30 seconds is followed by a small foot shock. Thus the animal learns to associate the tone with the shock. The amygdala-associated fear response caused by the tone is able to bypass the hippocampus once the association is made, allowing for assessment of emotionally driven memory formation. Surprisingly, no differences between wild type and FKBP5-/- mice were observed in either contextual (environmentally-based) or cued (tone-based) fear conditioning paradigms (Figure 2F-2H). These findings suggest that

neither spatially nor emotionally driven long-term memory is affected by FKBP5 deletion. In addition to these learning and memory tasks, no observable differences between wild type and FKBP5-/- mice were noted in tasks designed to assess activity, motor performance, motor coordination, motor learning, hearing, or prepulse inhibition, which measures startle response inhibition (Figure 3.3).

Brain-specific knockout of GR in mice reduces anxiety (Tronche et al., 1999). Also, FKBP51 expression was recently correlated to anxious behavior (Attwood et al., 2011). Based on this and the novel link now established between FKBP51, GR activity and glucocorticoid production, the effects of FKBP5 deletion on anxiety were tested. The longitudinal impact of FKBP5 deletion on anxiety was assessed in mice aged 11-14 months and then again in the same mice aged 18-22 months using the elevated plus maze (EPM). A repeated measures two-way analysis of variance was conducted to examine the effect of time and genotype on anxiety. The behavioral correlates of anxiety in the EPM were significantly affected by age, and this change depended on the genotype at FKBP5 as reflected by a significant age*genotype interaction, $F(1,14)=6.71$, $p=0.02$ (Figure 3.4 A&B). Indeed, in wild type mice behavioral correlates of anxiety decreased with age, whereas they appeared to increase in FKBP5-/- mice. To further

examine whether anxiety was indeed being modified in these mice, the 18-22 month-old mice were subjected to the light-dark chamber paradigm, which is another standard measure of anxiety. The mice were allowed to explore between a well-lit and unprotected area and a dark covered area that the mice had access to through a small opening. Normal mice typically move to the dark chamber. The light-dark maze did not reveal any statistical differences between the groups (Figure 3.4 C-E). These data suggest that suppression of FKBP51 may have a differential effect on anxiety depending on the developmental stage; however, the effect is not pervasive enough to manifest itself through several indices of anxiety.

Discussion

Major depression is a devastating disease with a course that is frequently chronic or recurrent and affects millions of people. Research in the last decade has shown that variation in the FKBP5 gene is associated with depression and several other mood and anxiety disorders. And although in vitro data suggests the possibility of a causal relationship between FKBP5 expression levels and depression, this has never been tested in vivo. Here we show for the first time that ablation of FKBP5 in mice led to reduced immobility in two behavioral

models that are routinely used to assess anti-depressant efficacy. This behavioral effect coincided with attenuation of corticosterone production after a stressful episode. Moreover, no defects in locomotion, somato-sensation or learning and memory were observed. Thus, therapies developed to reduce FKBP51 levels may be highly efficacious as next generation anti-depressants. Furthermore, because FKBP51 ablation results in reduced anxiety-like behavior in mice (Attwood et al., 2011), we provide experimental support for the notion that genetically-driven variations in expression of FKBP51 may underlie susceptibility to anxiety and mood disorders, as suggested by association studies in humans (Binder et al., 2004; Binder, 2009).

FKBP51 is a peptidyl-prolyl cis-trans isomerase (PPIase) enzyme that also associates with the chaperone Hsp90 and is distributed ubiquitously throughout the brain. This isomerase activity is thought to be important for structural rearrangements and phosphorylation dynamics of client proteins bound by Hsp90. Several diseases in addition to psychiatric conditions have implicated FKBP51 as having a role in their pathogenesis. These include prostate cancer (Ni et al.), and neurodegenerative diseases, specifically tauopathies (Jinwal et al., 2010). In fact, these effects on tau may somehow be tied to the manifestation of these psychiatric conditions. Indeed, depleting FKBP51 levels was shown to also reduce tau levels, while inhibiting its

PPIase activity actually lead to increased stability of phosphorylated tau. Thus it is certainly possible that FKBP51 is involved in Alzheimer's disease progression, since one of its earliest clinical features is depression. More recently, the extracellular protease neuropsin was shown to mediate anxiety-like behavior via an FKBP51 dependent mechanism (Attwood et al., 2011). Thus, an important role for FKBP51 in maintaining proper brain function is emerging. Its relationship with major depressive disorder in HIV, bipolar disorder and possibly anxiety and Alzheimer's disease further underlie its significance.

Current treatment for depression includes the use of medications that extend the amount of time neurotransmitters are present in the synaptic cleft including serotonin, norepinephrine, and dopamine. It is estimated that 60-70% of patients reach remission with the use of anti-depressant drugs (Rush et al., 2006). These low rates of efficacy have prompted research into other potential therapeutic targets in the HPA axis, particularly GR. However, there are many different isoforms of GR, making selective targeting with compounds challenging. Therefore, the results presented here show that FKBP51 may be the most appropriate target for treating depression via the modulation of the HPA axis in terms of its risk/benefit equation and potential therapeutic window. Also, and most noteworthy, because FKBP51 may act on the genetic liability to abnormal mood and anxiety states, it

may provide a much needed treatment tool for secondary prophylaxis of depression recurrence and relapse.

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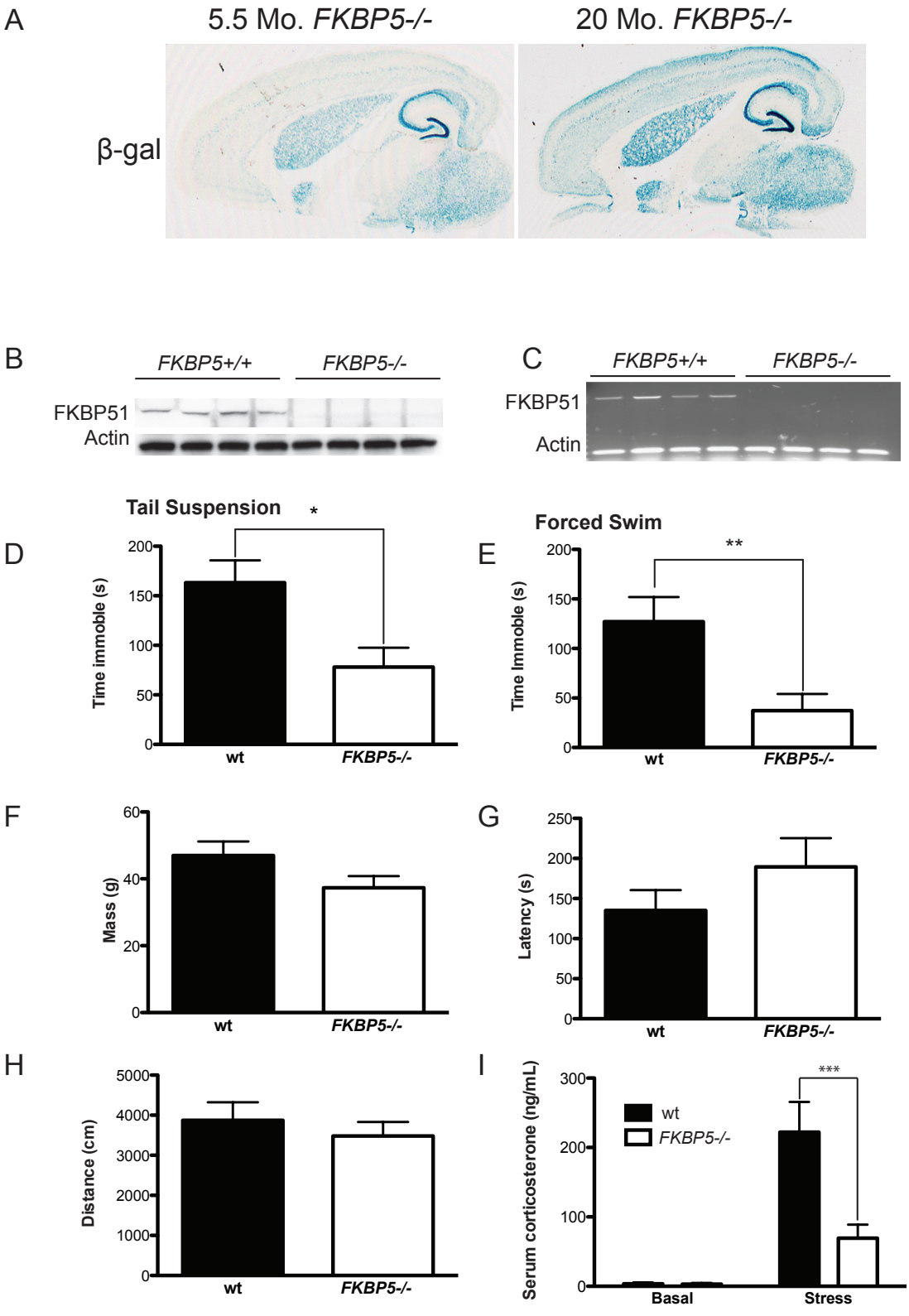
A
5.5 Mo. FKBP5-/-
20 Mo. FKBP5-/-
β-gal
B
FKBP5+/+
FKBP5-/-
FKBP51
Actin
C
FKBP5+/+
FKBP5-/-
FKBP51
Actin
D
Tail Suspension
*
Time immoble (s)
200
150
100
50
0
wt
FKBP5-/-
E
Forced Swim
**
Time Immoble (s)
200
150
100
50
0
wt
FKBP5-/-
F
Mass (g)
60
40
20
0
wt
FKBP5-/-
G
Latency (s)
250
200
150
100
50
0
wt
FKBP5-/-
H
Distance (cm)
5000
4000
3000
2000
1000
0
wt
FKBP5-/-
I
Serum corticosterone (ng/mL)
***
300
200
100
0
wt
FKBP5-/-
Basal
Stress

Figure 3.1

Figure 1. *FKBP5-/-* mice display resistance to depression inducing stimulus. (A) Representative images of β-gal staining in horizontal brain slices of 5.5 and 20 month old *FKBP5-/-* mice. (B) Western blot analysis of wild type and FKBP5-/- whole brain homogenates. (C) *FKBP5* primer-specific PCR of cDNA synthesized from brain-isolated mRNA by reverse transcription. (D) Total time immobile in the tail suspension test. (E) Total time immobile in the forced swim test. (F) No significant difference was observed between mass measurements of groups. (G) Latency to fall from a gradually accelerating rotorod apparatus displays no difference between groups. (H) Activity levels in the open field display no differences between groups. (I) Levels of corticosterone in serum right before a 10-minute tube restraint and 30 minute after (wt n=5, *FKBP5-/-* n=6). * $p<0.05$, ** $p<0.01$.

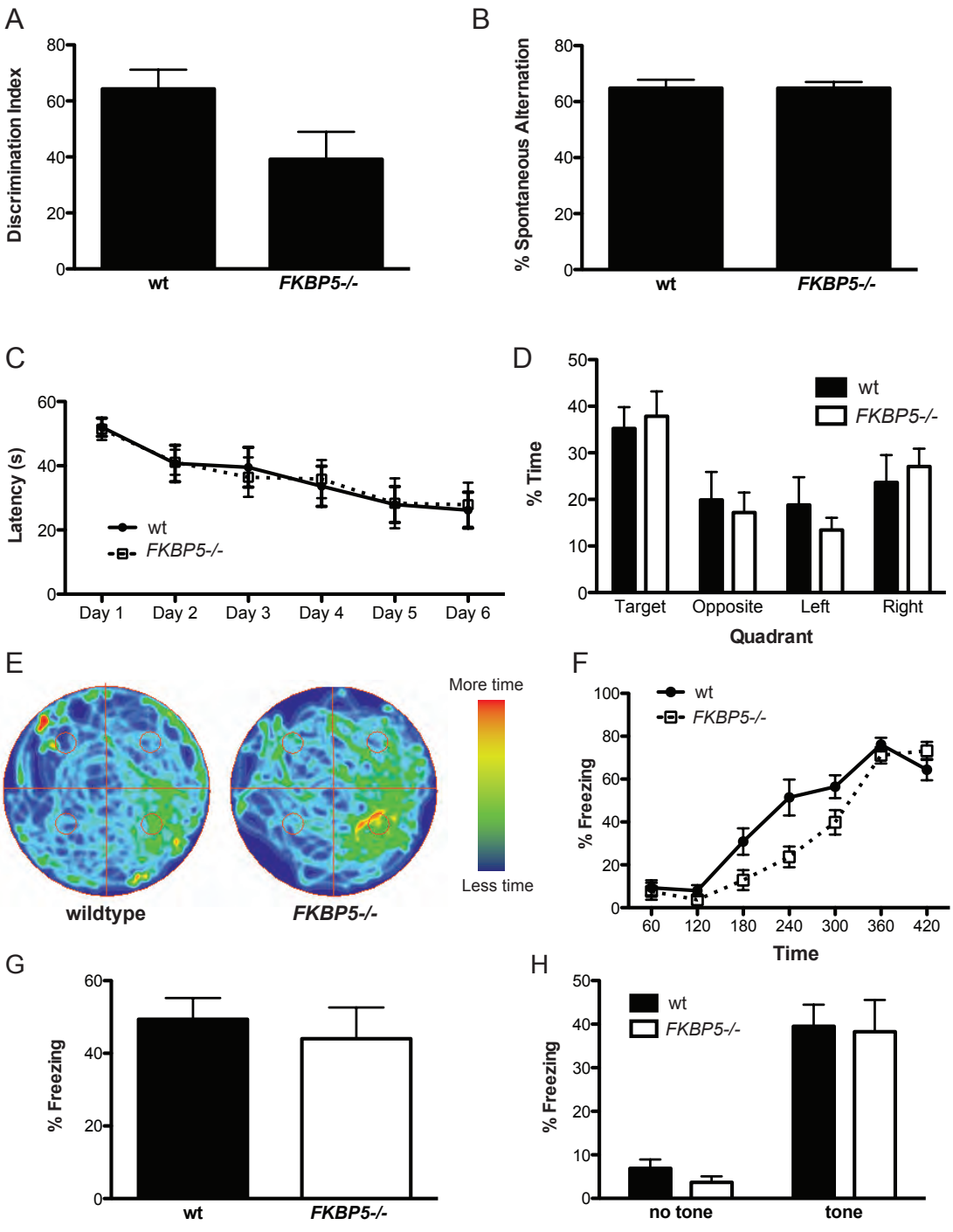
A
Discrimination Index
80
60
40
20
0
wt
FKBP5-/-
B
% Spontaneous Alternation
80
60
40
20
0
wt
FKBP5-/-
C
Latency (s)
60
40
20
0
wt
FKBP5-/-
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
D
% Time
50
40
30
20
10
0
wt
FKBP5-/-
Target
Opposite
Left
Right
Quadrant
E
More time
Less time
wildtype
FKBP5-/-
F
% Freezing
100
80
60
40
20
0
wt
FKBP5-/-
60
120
180
240
300
360
420
Time
G
% Freezing
60
40
20
0
wt
FKBP5-/-
H
% Freezing
50
40
30
20
10
0
wt
FKBP5-/-
no tone
tone

Figure 3.2

Figure 2. Deletion of the FKBP5 gene does not alter learning and memory despite robust expression of FKBP5 in the hippocampus. (A) Discrimination index between familiar and novel object in the novel object recognition test. (B) Percent of spontaneous alternation shows no difference between the groups. (C) Learning of the location of the hidden platform in the MWM (interaction $F(5,80)=0.14$, $p=0.98$; genotype $F(1,16)=0.0$, $p=0.98$; time $F(5,80)=12.71$, $p<0.0001$) . (D) Percent time spent in quadrant during the probe trial of MWM. (E) Software generated heat plot representing amount of time spent in an area. (F) Percent freezing during training portion of fear conditioning (interaction $F(6,90)=1.32$, $p=0.26$; genotype $F(1,15)=0.01$, $p=0.92$; time $F(6,90)=14.35$, $p<0.0001$). (G) Percent freezing during contextual exposure to fear-conditioning chamber. (H) Percent freezing during the cued fear-conditioning test.

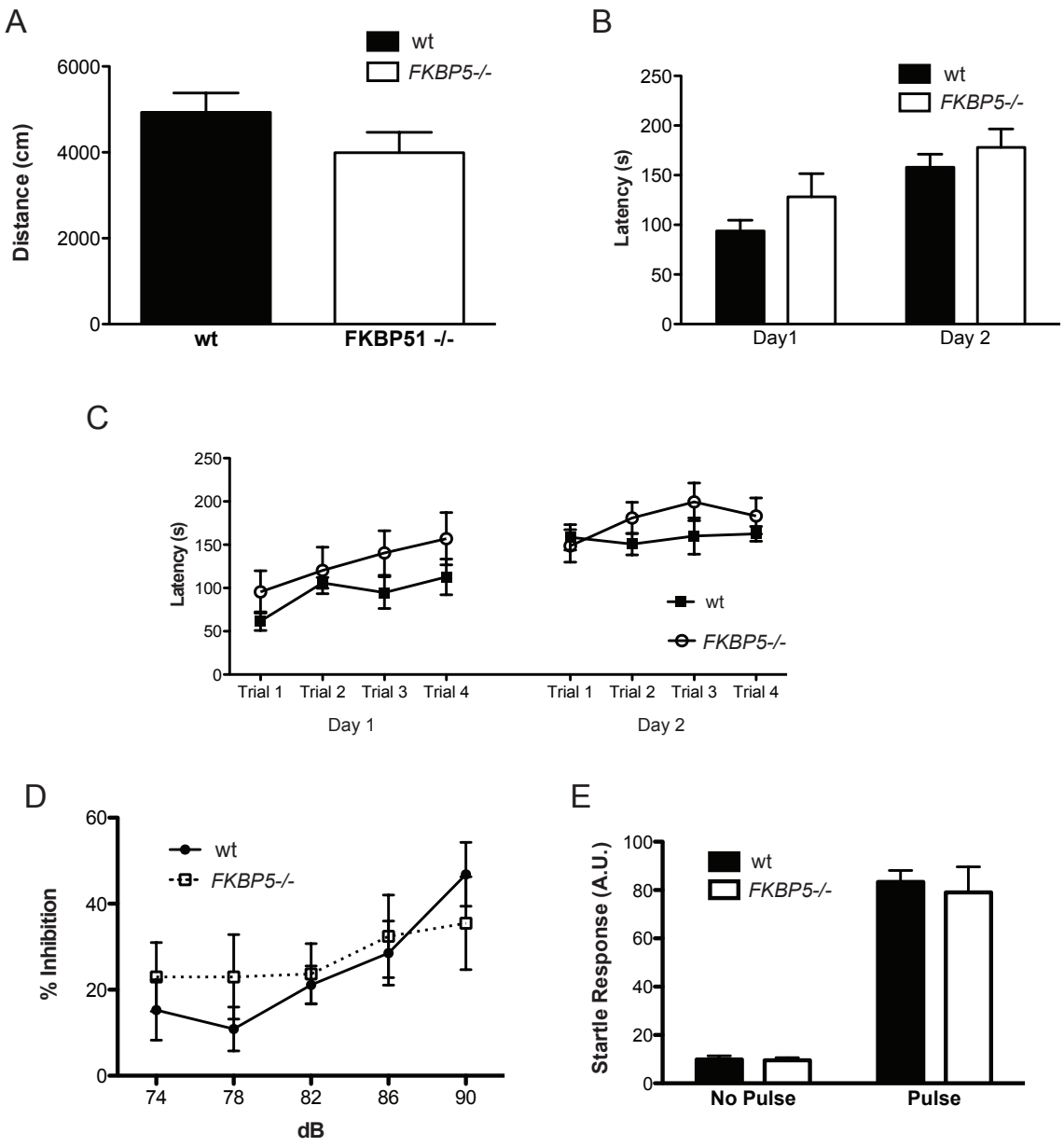
A
wt
FKBP5-/-
Distance (cm)
6000
4000
2000
0
wt
FKBP51 -/-
B
wt
FKBP5-/-
Latency (s)
250
200
150
100
50
0
Day1
Day 2
C
Latency (s)
250
200
150
100
50
0
Trial 1
Trial 2
Trial 3
Trial 4
Day 1
Trial 1
Trial 2
Trial 3
Trial 4
Day 2
wt
FKBP5-/-
D
wt
FKBP5-/-
% Inhibition
60
40
20
0
74
78
82
86
90
dB
E
wt
FKBP5-/-
Startle Response (A.U.)
100
80
60
40
20
0
No Pulse
Pulse

Figure 3.3

Figure 3.3. Behavioral characterization of FKBP5-/- mice. (A) Distance traveled in the open field test. (B) Assessment of motor learning from comparison between the average of all four trials of rotorod from day one with those of day two. (C) Latency to fall from the rotorod apparatus. (D) Percent inhibition of startle response by increasing prepulse intensity. (E) Comparison of no pulse (no tone) with a 120 dB pulse (tone) to test hearing.

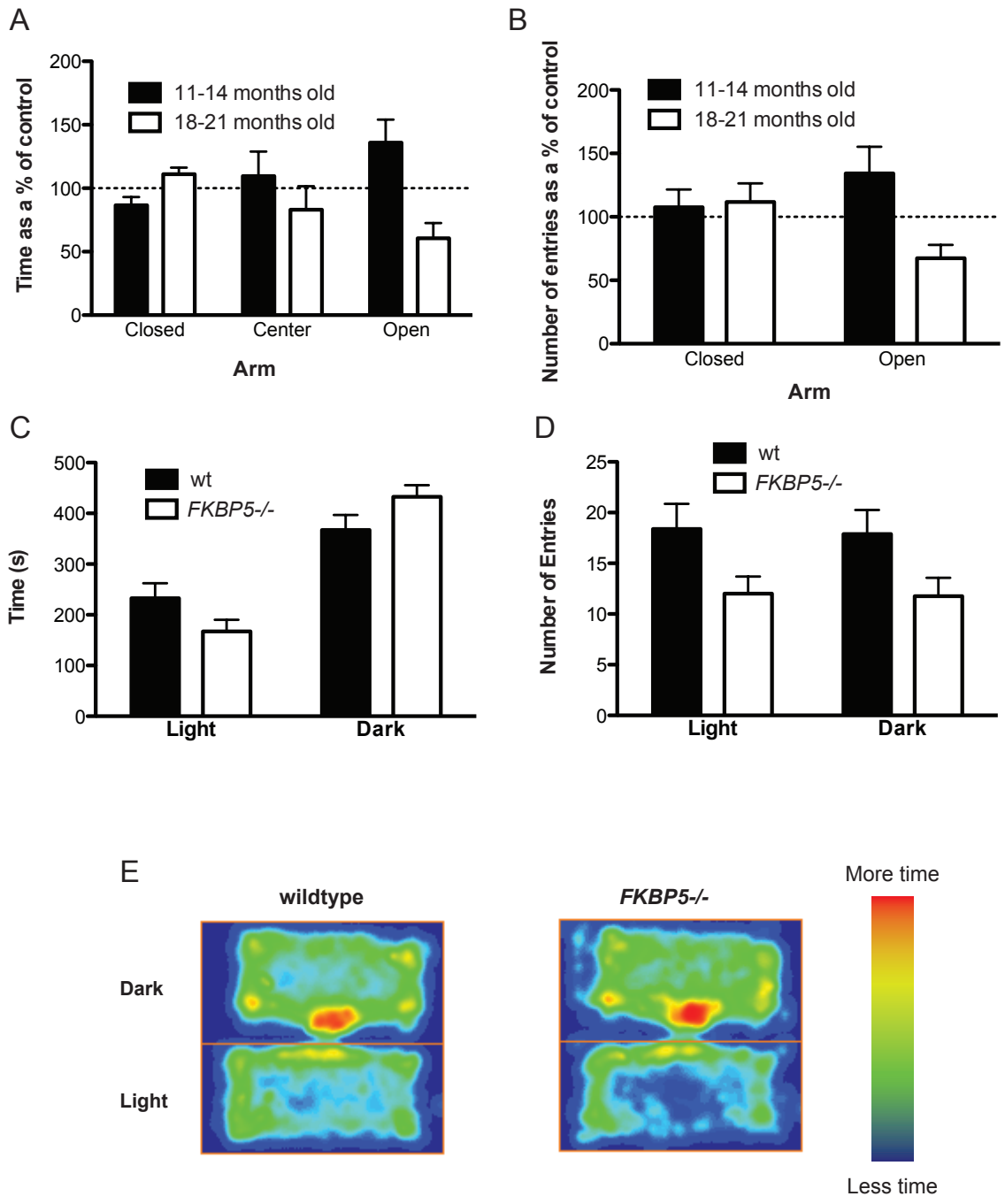
A
Time as a % of control
200
150
100
50
0
11-14 months old
18-21 months old
Closed
Center
Open
Arm
B
Number of entries as a % of control
200
150
100
50
0
11-14 months old
18-21 months old
Closed
Open
Arm
C
Time (s)
500
400
300
200
100
0
wt
FKBP5-/-
Light
Dark
D
Number of Entries
25
20
15
10
5
0
wt
FKBP5-/-
Light
Dark
E
wildtype
FKBP5-/-
Dark
Light
More time
Less time

Figure 3.4

Figure 3.4. Age-associated changes in anxious behavior in FKBP5-/- mice. (A) Time spent in the arms of the EPM as a percentage of the control group (wt and FKBP5-/- 11-14 mo. n=9, wt and FKBP5-/- 18-21 mo. n=8). (B) Number of entries into the arms of the EPM as a percentage of control. (C) Time spent in the areas of the dark-light chamber (wt n=8, FKBP5-/- n=8). (D) Number of entries into the areas of the dark-light chamber (wt n=8, FKBP5-/- n=8). (E) Software generated heat plot representing amount of time spent in area. *p<0.05, **p<0.01, ***p<0.001.

CHAPTER FOUR:

FKBP5-/- Enhances Cognitive Flexibility, and Protects From Age and Genotype Dependent Loss of Resilience.

Abstract

The ability to respond effectively to, and recuperate from stressful events is a key aspect of survival, and emotional health. However, it is not clear if there is a reduction in stress resiliency with age, and particularly, how age-related changes in gene expression in the Hypothalamic-Pituitary-Adrenal axis modulate the response to stress. Moreover, there is a relationship between memory and resiliency, but it is not known how. In particular, it is not known if there are proteins that regulate resiliency and memory. Here we show that wild type mice display decreased stress resiliency with age in the tail suspension test, a behavioral pattern of resignation and loss of effort that can be modulated with antidepressants. They also display greater serum corticosterone levels after stress with age. The FKBP5

gene, which codes for a hypothalamic-pituitary-adrenal-axis regulating protein, may be partly responsible for this age-dependent decrease in resiliency, as FKBP5-/- mice display protection from stress and age induced despair. Deletion of this protein provides wild type mice with greater cognitive flexibility in the reversal of the 6 radial arm water maze. In addition, the absence of this protein is not deleterious to the lifespan or healthspan of the mice, suggesting that this gene is a possible therapeutic target for stress and aging induced psychiatric disease.

Introduction

Resilience is the ability to recover and to spring back into shape after difficulties, to have elasticity (Russo et al., 2012). Although there are myriad environmental factors that have been shown to help patients recover from traumatic events, like strong social networks (Ozbay et al., 2007; Ozbay et al., 2008), positive outlook (Black and Lobo, 2008; Schmiege et al., 2011), and access to mental health professionals (Dowrick et al., 2009), several biological factors have also been found to be involved in resiliency, like patterns of brain activity (Shin et al., 2005), signaling molecules (Morgan et al., 2000),

genes (Binder et al., 2008), and epigenetic mechanisms (Klengel et al., 2012).

Several genes have been singled out for their effect on stress resiliency in animals and humans, and one of them is the FK506-Binding Protein 5 (FKBP5) (O'Leary et al., 2011; Touma et al., 2011; Hartmann et al., 2012). People who experience childhood trauma, like physical and sexual abuse, who carry certain FKBP5 single nucleotide polymorphisms (SNPs), are predisposed to develop PTSD (Binder et al., 2008), major depressive disorder (MDD) (Zimmermann et al., 2011), and suicide (Brent et al., 2010; Roy et al., 2010; Supriyanto et al., 2011; Roy et al., 2012). One particular SNP, rs1360780, has been shown to increase the levels of FKBP5 in the cell and cause long-lasting epigenetic effects that further increase FKBP5 levels, providing a plausible mechanism for gene x environment interactions (Binder et al., 2004; Klengel et al., 2012). In mice, knockdown of FKBP5 in the amygdala produces an anxiolytic effect after stress (Attwood et al., 2011), and global knockout of this gene increases resiliency to the forced swim, tail suspension (O'Leary et al., 2011; Touma et al., 2011), and chronic social defeat stress (Hartmann et al., 2012). FKBP5 is expressed widely throughout the brain, but its role has been mostly studied within the Hypothalamic-Pituitary-Adrenal (HPA) axis (Barik,

2006). It is a regulator of the glucocorticoid receptor (GR), as it reduces the affinity of glucocorticoids for the GR, negatively impacting its activity (Reynolds et al., 1999; Denny et al., 2000; Scammell et al., 2001; Denny et al., 2005; Wochnik et al., 2005). GR activity is extremely important protein, as global knockout of the gene is lethal perinatally (Cole et al., 1995). In particular, GR is responsible for the shut-off of the HPA-axis (Tasker and Herman, 2011). Consequently, by inhibiting the GR, FKBP5 allows for the HPA-axis to be turned on for a longer period of time, producing greater amounts of glucocorticoids. Hyperglucocorticoidism is common in depression (Herbert, 2013), and it is also linked to the perception of anguish during stress. A study that subjected active duty personnel to 24 hours of U.S. Navy survival school stress, revealed that distress perception was significantly correlated to cortisol expression (Morgan et al., 2002).

However, what happens to our emotional elasticity as we age? Although very little is known about the longitudinal effects of resiliency, research in the aging brain suggests that it is more susceptible to neurodegenerative and psychiatric diseases, with Alzheimer's and depression in particular (Sibille, 2013). This leads to the speculation that the brain has reduced resiliency with age. Moreover, several of the disease processes involved in aging,

neurodegeneration, and mental illness, share pathological commonalities like inflammation, dysfunction in the HPA axis, and metabolic syndrome. This makes it conceivable that age-dependent gene expression may play an important role.

Memory, like resilience, is also modulated by age, emotion, and stress, and may play a role in resilience. An overactive amygdala has been shown as a feature of PTSD (Shin et al., 2011). Presumably, excessive amygdaloid activity enhances emotional memory formation, increasing the effect of a traumatic event. Another protein involved in resiliency, anxiety, and psychiatric disorders, NPY, has also been implicated in the acquisition, consolidation and extinction of memory (Lach and de Lima, 2013). Conceivably, memory strength is important for emotional processing of events. FKBP5 is expressed in virtually all of the brain regions involved with memory. In particular, the hippocampus, the amygdala, and prefrontal cortex (O'Leary et al., 2011; Scharf et al., 2011). However, a previous study showed no impact in the ability to form new memories in the FKBP5-/- mice (O'Leary et al., 2011).

FKBP5 has recently been found to be expressed age-dependently in humans and mice. This, in combination with its stress inducement

and the clinical studies implicating it with disease, places FKBP5 in a unique position to be capable of being an age-dependent enabler for reduced stress resiliency. In addition, its role in resiliency and its topography in the brain indicate a possibility of altered memory processing, since FKBP5 is present in all the regions of the brain that handle memory. Here, we aged C57BL/6 mice and longitudinally tested their ability to respond to the forced swim and tail suspension tests. We found that wild type mice display reduced resiliency in the tail suspension test with age, and the FKBP5-/- mice display increased resiliency due to genotype and aging. Moreover, the FKBP5-/- display reduced serum corticosterone after stress at all ages, and the wild type mice produce increased serum corticosterone after stress with age. Additionally, the FKBP5-/- mice display greater cognitive flexibility, as they learn the reversal of the hidden platform faster than the wild type mice. Moreover, the FKBP5-/- mice have equal rates of survival as wild type mice up to 800 days. These data suggest that FKBP5-/- is beneficial for resiliency and cognitive flexibility, and that reduction of FKBP5 is a promising therapeutic strategy.

Materials & Methods

FKBP5-/- mice

The mice have been generated as previously described (O'Leary et al., 2011) (Touma et al., 2011). Briefly, by PCR screening, the 129SvJ mouse BAC library (Genome Systems, St. Louis, MO), and the bacterial artificial chromosome (BAC) clones that contained genomic regions for FKBP5 were isolated. Restriction fragments were subcloned into pBluescript (pBS; Stratagene, La Jolla, CA) or pZero (Invitrogen, Carlsbad, CA) cloning vectors, and the PCR products were amplified from the BAC clones, which were then used to construct a targeting vector in the pPGKneo vector (a generous gift of James Lee, Mayo Clinic Scottsdale). The targeting vector contained a beta-galactosidase/neomycin cassette flanked by regions homologous to the FKBP5 gene. Due to the size of the protein it is more practical to partially delete the gene. Consequently, when the targeting vector integrates into the chromosome through homologous recombination it removes all of exon 2, which is the first coding exon. Since the only deleted portion of the gene is exon 2, the expression of the beta-galactosidase protein is dependent on the FKBP5 promoter and transcription machinery, and expresses in frame with the initiation codon. ES cells were isolated from the 129SvJ mouse and cultured in

Knockout DMEM media (Invitrogen) supplemented with 10% FBS, penicillin/streptomycin, essential amino acids, and ESGRO (103 U/ml; Chemicon, Temecula, CA) with irradiated embryonic fibroblast feeder cells. The ES cells were then electroporated at 0.2 kV, 950 μF (Gene Pulser II; Bio-Rad, Hercules, CA) with linearized targeting vectors and selected with G418. DNA from G418-resistant clones was isolated for Southern blot analysis, and a DNA probe was used to distinguish PstI restriction fragments from wildtype allele (~7.5 kb) and targeting vector (~10 kb). Appropriate homologous recombination in ES cell clones was confirmed by PCR using primers complementary to sequences within the neomycin cassette and to 3' FKBP5 sequences downstream from the recombination site. ES cell clones containing the targeting vector were injected into C57BL/6 blastocysts and implanted into pseudopregnant 129SvJ females. Chimeric offspring were identified by coat patterns and mated to C57BL/6 mice to obtain germline transmission of the targeting vector. For colony maintenance mice were crossed from C57BL6 onto Swiss-Webster for purposes of fecundity and genetic diversity to be more representative of a human population.

Porsolt forced swim test (FST)

This test was performed as previously published (O'Leary et al., 2011). Each mouse was placed in a tall (45 centimeters high and 20 centimeters diameter) clear Plexiglas cylinder filled with room temperature water to a depth of 12 centimeters for a total of 6 minutes. The total time immobile was measured. Any immobility episode below two seconds was not used towards the total.

Tail suspension test (TST)

Each mouse was suspended from the tail for 6 minutes. The total time spent immobile was measured. Any immobility episode below two seconds was not used towards the total.

Radial arm water maze

A six arm apparatus was placed inside a black painted pool, filled with 10 cm of water. The pool was close to the room’s walls for queue visibility. The hidden platform was located in a different arm for each mouse (goal arm), and the arm in which they were placed at the beginning of each trial (start arm) was changed every trial. Each mouse had 60 seconds to find the hidden platform in any given trial. The number of incorrect arm entries and the escape latencies were recorded. An entry is considered as such when all four limbs have

entered the arm. An error was also scored when the mouse did not choose an entry for 15 sec. If the mouse did not find the platform after 1 minute, the mouse was gently guided to and placed on it. Afterwards, the mouse was kept on the platform for 15 seconds. The mice were towel dried and placed under a heat lamp to dry completely. On day one, trial one through twelve alternated between visible and hidden platforms, and trial thirteen through fifteen were performed using the hidden platform only. A total of fifteen trials a day were performed. On day two, only the hidden platform was used. On day four, a goal arm reversal was performed, where the hidden platform was placed in the directly opposite arm. The same procedure was repeated for the revearsal, for a total of six days of training: three days for initial platform training and three days for retraining. This protocol is based on a previously published one (Alamed et al., 2006).

Corticosterone assay, blood collection and stress paradigm

The levels of corticosterone were measured using an ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA). Blood from the mice was collected in the morning one hour after the light cycle began and 30 min after a 10-min tube restraint using the submandibular vein puncture method.

Glucose challenge

Mice were fasted overnight. The next morning they were injected with a 2g/kg dose of sucrose (Sigma) into the peritoneum. Blood drops were collected and analyzed in a standard glucometer at baseline, 30, 75, and 120 minutes after injection.

Western blotting and tissue processing

Western blotting was performed as previously described (Dickey et al., 2006). Briefly, mouse brain samples were homogenized in lysis buffer containing 50 mM Tris-HCl pH 8.0, 274 mM NaCl, 5 mM KCl, phosphatase inhibitor cocktail I (Sigma), phosphatase inhibitor cocktail II (Sigma), mammalian protease inhibitor cocktail (Sigma), and 1 mM PMSF (Sigma). Protein concentration was determined using a bicinchoninic acid protein assay (Peirce). Samples were heated for five minutes in Laemmli's buffer and 5% B-mercaptoethanol (Sigma). Equal amounts of protein were loaded onto 10% Tris-glycine SDS-PAGE gels. Afterwards, the gels were transferred onto polyvinalidene fluoride membranes (Millipore) using the Biorad transfer system, and blocked for 1 hour in 5% non-fat dry milk diluted in Tris buffered Sailne and 0.2% Triton-X 100 (Fischer).

Complete blood count

Complete blood count measurements were performed with a blood analyzer (Heska).

Size measurements

Size measurements were performed using a standard digital caliper.

Cytokine measurements

Cytokine levels were measured using the Bioplex system from Biorad with the Biorad 8-panel cytokine kit.

Long-term potentiation

LTP was performed as previously described (Abisambra et al., 2010). For the stress experiment, mice were restrained in their cages for 1 hour.

Statistics and chance levels

Assuming that the mice can adjust their performance to make no more than 6 errors, only entering an arm once, we can determine the chance level using a a negative hypergeometric distribution with the following equation, (ɑ(n + 1)/(ɑ + 1)) - ɑ. "ɑ" is the total number

of arms with an escape (platform), and "n" is the total number of arms. This results in 2.5. This was previously published (Diamond et al., 1996; Diamond et al., 1999).

Results

FKBP5-/- mice have been shown to display stress resiliency (O'Leary et al., 2011; Hartmann et al., 2012). At three months of age the amount of stress required to precipitate this phenotype was much greater than at 21 months of age, since they required prior restraint stress to display a phenotype in the forced swim test, and 21 month old mice do not. However, age also increases the levels of FKBP5 (Blair et al., In Press)(Jinwal et al., 2010; O'Leary et al., 2011). Consequently, we wanted to investigate if there is a relationship between age and behavior due to FKBP5. We subjected two cohorts of FKBP5-/- mice and their wild type littermates ages 6, and 21 months to the tail suspension and forced swim tests. In the tail suspension test, analysis by a two-way ANOVA revealed that there was a strong genotype ($F(1,30)=13.30$, $^{***}p=0.001$), and aging effect ($F(1,30)=10.33$, $^{*}p=0.00031$) for increased resilience in the FKBP5-/- mice (Figure 4.1 A). In the forced swim test, there was a strong

genotype effect ($F(1,33)=14.12$, $p=0.0007$), but no aging effect ($F(1,33)=0.70$, $p=0.4102$) (Figure 4.1 B).

Due to the strong inhibition FKBP5 confers on GR we also measured serum corticosterone levels after stress longitudinally (Figure 4.2). There is a strong genotype effect for the FKBP5-/- mice to produce less corticosterone after stress ($F(1,50)=13.26$, ***$p=0.0006$). There is also a significant effect for the increase in corticosterone with age ($F(1,50)=4.46$, *$p=0.0397$) which the FKBP5-/- mice are protected from.

Glucocorticoids are very important for general metabolic processes and immune system function. They are known to promote fat deposition and glucose metabolism (Lee et al., 2013). Glucocorticoids are given routinely to reduce inflammation (Barnes, 1998). Since these mice produce less glucocorticoids during stress, we tested their weight, and size. There were no significant changes between these (Table 4.1). To test for glucose metabolism we challenged these mice with a 2g/kg of sucrose injected into the peritoneum after an overnight fast. We tested blood levels at 0 (before injection), 30, 75 and 120 minutes after injection. There was no significant change in glucose metabolism in these mice (Figure 4.3).

FKBP5 is actually primarily known as an immune protein, since it is part of a family of proteins that provide immunosuppressive properties to FK506 and cyclosporine. Because of this and the corticosterone phenotype, we also tested for changes in the number of white blood cells, and there were no differences between the groups (Table 4.1).

Glucocorticoids also have important immunosuppressive properties. Since FKBP5-/- mice produce less corticosterone after stress, we tested the effect of this hypoglucocorticoid phenotype on the levels of serum cytokines at different ages (Figure 4.4). We found that at 7 months FKBP5-/- mice display a reduction in IL-1β, and IL-5. However, this difference is not present at 10 and 22 months old.

We, and others, have previously shown that FKBP5 is highly expressed in the hippocampus (O'Leary et al., 2011; Scharf et al., 2011). Consequently, we previously performed the Morris water maze on the FKBP5-/- mice to test if the ablation of the gene had any effect on learning and memory. Despite its robust expression in this highly important memory center, there were no changes. Even so, It may be that the absence of FKBP5 has an effect in other regions of the brain involved with memory. The radial arm water maze offers an advantage for this question, since the mice are forced to make a decision as to which arm they should enter. Decision-making requires a group of

brain regions working together (Clark et al., 2004). Using a reversal experiment, where the platform is changed to an alternative arm, the mice have to relearn the position of the platform. Consequently, we can test for cognitive flexibility and the ability to relearn. We did not observe any changes between the initial acquisition of the of RAWM training (Figure 4.5 A). When we reversed the location of the platform, however, the FKBP5-/- mice were better able to relearn (Figure 4.5 B). They quickly reached the learning criteria. This criteria consists of committing fewer errors than chance, which is 2.5 errors per trial in this paradigm, as established previously (Diamond et al., 1996). The FKBP5-/- mice achieved this on trial 18 (day four), but the wild type mice did not cross this threshold until trial 21 (day five). The total number of errors per day shows no change in the first three days (Figure 4.5 C), but the reversal displays a significant genotype effect ($F(1,16)=4.9$, $p=0.0417$), as the knockout mice learn the new location faster, and make fewer errors (Figure 4.5 D). Despite the greater ability to display cognitive flexibility, there is no change in hippocampal long-term potentiation (Figure 4.6 A), suggesting this could be an enhancement from another brain region, perhaps the prefrontal cortex. There is also no significant difference between the slopes of the amplitude of the fiber volley and the slopes of the field excitatory post-

synaptic potential, which indicates a normal relationship between the presynaptic and postsynaptic response (Figure 4.6 B).

One potential role for FKBP5 is to protect from GR activity, since the hippocampus is very sensitive to glucocorticoids (Popoli et al., 2012). To test for this we challenged 12 month old wild type and FKBP5-/- mice to acute restraint stress for one hour prior to the performance of long term potentiation. We found that FKBP5-/- mice display reduced potentiation and do not maintain long term potentiation as compared to the wild type mice (Figure 4.7 A). There is a significant genotype effect $F(1,624)=75.47$, $^{***}p<0.0001$), and time effect ($F(29,624)=3.95$, $^{***}p<0.0001$). The last five minutes are also significantly different $F(1,105)=39.24$, $^{***}p=0.0001$). This difference is not altered due to an altered postsynaptic response due to a presynaptic stimulus(Figure 4.7 B).

Due to the strong genotype effect in resilient behavior and corticosterone signaling, FKBP5 is a promising therapeutic target for the treatment of psychiatric disease and emotional endurance. As a result, it is important to study the effect of longterm FKBP5 ablation on the lifespan. We aged wild type and FKBP5-/- mice up to ~800 days and both populations displayed 50% survival of the cohort (Figure

4.8). There was no statistical significance between the groups (Log-rank, Mantel-Cox, test, χ^2=0.001354, p=0.9707).

Discussion

Here we show that FKBP5 has an effect in resiliency with aging and genotype and that its ablation is beneficial in responding to emotionally stressful situations. We also show that the lack of FKBP5 allows for greater cognitive flexibility in non-emotional memory, as the FKBP5-/- mice quickly adapt to the reversal of the hidden platform in the 6 radial arm water maze. Moreover, the lack of FKBP5 does not affect the lifespan or healthspan of these mice.

One interesting observation from these data is that there is an age dependent increase in immobility in the tail suspension test that is not present in the forced swim test. From observation of the videos of the mice in these tests, it is plausible that the tail suspension test is a more uncomfortable test than the forced swim test, as they seem to struggle more. In the forced swim test the water is at room temperature, ~23-25 , and although the mice do not like swimming, they are very good swimmers. Thus, the level of discomfort may be less than the tail suspension test. This could explain the discrepancy

between the two tests. However, there is still a strong genotype effect regardless of aging.

Because glucocorticoids promote anti-inflammation, and the FKBP5-/- mice display hypoglucocorticoidism after stress, we expected the levels of basal cytokines to increase in these mice with age. However, this did not occur. In fact, the exact opposite was observed for the 7-month old cohort. One possible explanation is that the chronic absence of FKBP5 causes the activation of a compensatory mechanism for inflammation.

Previously, the FKBP5-/- mice did not exhibit any hippocampal memory deficits (O'Leary et al., 2011), and here we show no change in hippocampal LTP, proving that there is no abnormalities to the hippocampus in the FKBP5-/- mice. As a result, we tested these mice in the radial arm water maze, as the mice are required to make a decision. Decision-making is dependent on several brain regions working together (Clark et al., 2004; Euston et al., 2012). This may be better able to show if there is a change in memory processing as a whole. The results here show that FKBP5 reduces cognitive flexibility. Interestingly, it has been shown that in depression, patients also display reduced cognitive flexibility (Deveney and Deldin, 2006;

Murphy et al., 2012). The prefrontal cortex has been identified as an important brain region for cognitive flexibility (Clark et al., 2004; Armbruster et al., 2012). This area has also been identified as under active in patients with PTSD (Shin et al., 2006), and it is also involved in resilience, since its activity is enhanced in people who experience trauma, but do not develop PTSD (Fani et al., 2012). These resilient subjects also display stronger physical connections in the circuitry connecting the hippocampus with the anterior cingulate cortex, an area of the prefrontal cortex. PTSD-subjects also display greater amygdaloid activity (Shin et al., 2006). In context with these data, FKBP5 deletion may manifest its effects by reducing amygdaloid activity and increasing prefrontal cortex activity during or even after a stressful event. Mice treated with an shRNA virus against FKBP5 in the amygdala display reduced anxiety in the elevated plus maze, after six hours of restraint stress (Attwood et al., 2011). In the same study, N-methyl-D-aspartate receptor activity in the Amygdala resulted in a massive increase in FKBP5, which led to anxious behavior.

Astonishingly, in all the literature regarding FKBP5, we have found only a few benefits to having this protein. Patients with the FKBP5 SNP rs1360780 display a more rapid response to antidepressant treatments compared to other depressed patients without this FKBP5

allele (Binder et al., 2004). This includes treatment with selective serotonin reuptake inhibitors, tricyclic antidepressants, and mirtazapine. However, these same patients have a greater number of depressive episodes throughout their life. The aging data in this study further presses the point that the lack of this gene is not damaging, and may even be beneficial.

However, it has been shown that acute stress enhances memory (Shors, 2001; Spyrka et al., 2011). Here, we show that FKBP5-/- mice are not able to display enhanced LTP due to acute stress. As a result, it is possible that FKBP5 protects the hippocampus from damage due to excessive glucocorticoid receptor activity. It may be that chronic stress given to the FKBP5-/- mice may damage the hippocampus to a greater extent than wild type mice. This would be a good future study.

Conclusion

In conclusion, this study shows that FKBP5 knockout animals are protected from an age and genotype dependent reduction in resilience, and increased corticosterone production. Moreover, FKBP5 knockout provides greater cognitive flexibility, and does not reduce lifespan. As a

result, FKBP5 may be an excellent target for the treatment of psychiatric diseases of the elderly.

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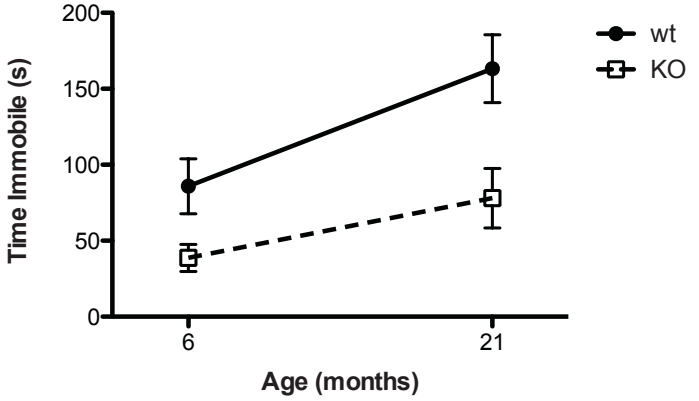
Table 4.1. FKBP5-/- mice display no changes in body weight, size, or white blood cell counts.

		Wild type			FKBP5-/-			
	Units	Value	S.D.	N	Value	S.D.	N	P value
Mass	g	43.04	11.6	12	38.82	8.382	13	0.3043
Width	mm	26.78	7.674	12	24.35	4.294	13	0.3338
Depth	mm	23.94	6.734	12	21.57	3.88	13	0.2868
WBC	10^3/uL	4.155	2.282	11	3.3	2.854	12	0.4394

WBC, white blood cells; S.D., standard deviation; N, sample size. P-value was derived by t-test comparing wild type with FKBP5-/- animals.

A


200
150
100
50
0
Time Immobile (s)
6
21
Age (months)
wt
KO

B

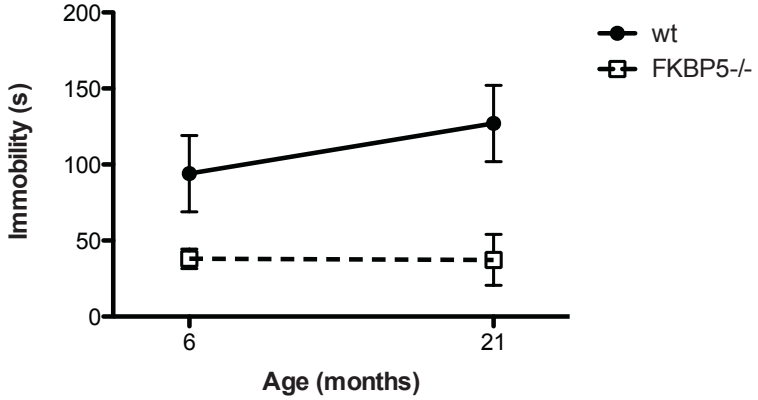

200
150
100
50
0
Immobility (s)
6
21
Age (months)
wt
FKBP5-/-

Figure 4.1

Figure 4.1. FKBP5-/- mice display a protection from FKBP5-induced decrease in resiliency. (A) Amount of time spent immobile in the tail suspension test in three separate cohorts of mice at two different ages. Analysis by 2-way ANOVA revealed a genotype effect ($F(1,30)=13.30$, ***$p=0.001$), aging effect ($F(1,30)=10.33$, *$p=0.00031$). (B) Amount of time spent immobile in the forced swim test in three separate cohorts of mice at three different ages. Analysis by 2-way ANOVA revealed a genotype effect ($F(1,33)=14.12$, $p=0.0007$), but no aging effect ($F(1,33)=0.70$, $p=0.4102$).

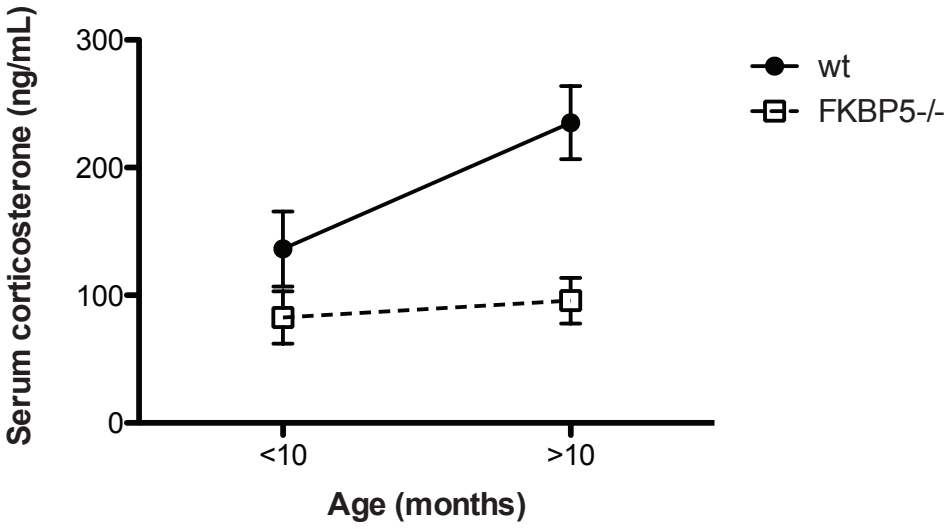

Serum corticosterone (ng/mL)
300
200
100
0
<10
>10
Age (months)
wt
FKBP5-/-

Figure 4.2

Figure 4.2. FKBP5-/- mice exhibit reduced serum corticosterone after stress, which is exacerbated with age. Several cohorts of mice were tested in this experiment, n=25 wt, n=29 FKBP5-/-. Post hoc, the mice were plotted by groups according to the age of 10 months. Serum corticosterone after stress shows a strong genotype effect ($F(1,50)=13.26$, ***$p=0.0006$). Serum corticosterone after stress also increases with age, ($F(1,50)=4.46$, *$p=0.0397$).

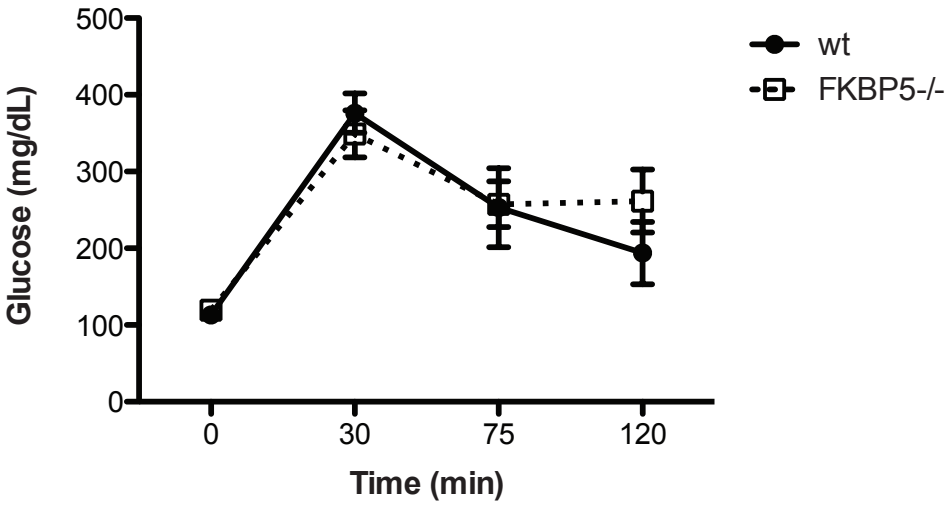


Figure 4.3

Figure 4.3. FKBP5-/- mice display normal glucose processing. Glucose challenge of wild type and FKBP5-/- mice. Mice were fasted overnight and challenged the next morning with a 2 g/kg dose of sucrose and their glucose levels were monitored for two hours ($F(1,15)=0.17$, $p=0.6855$).

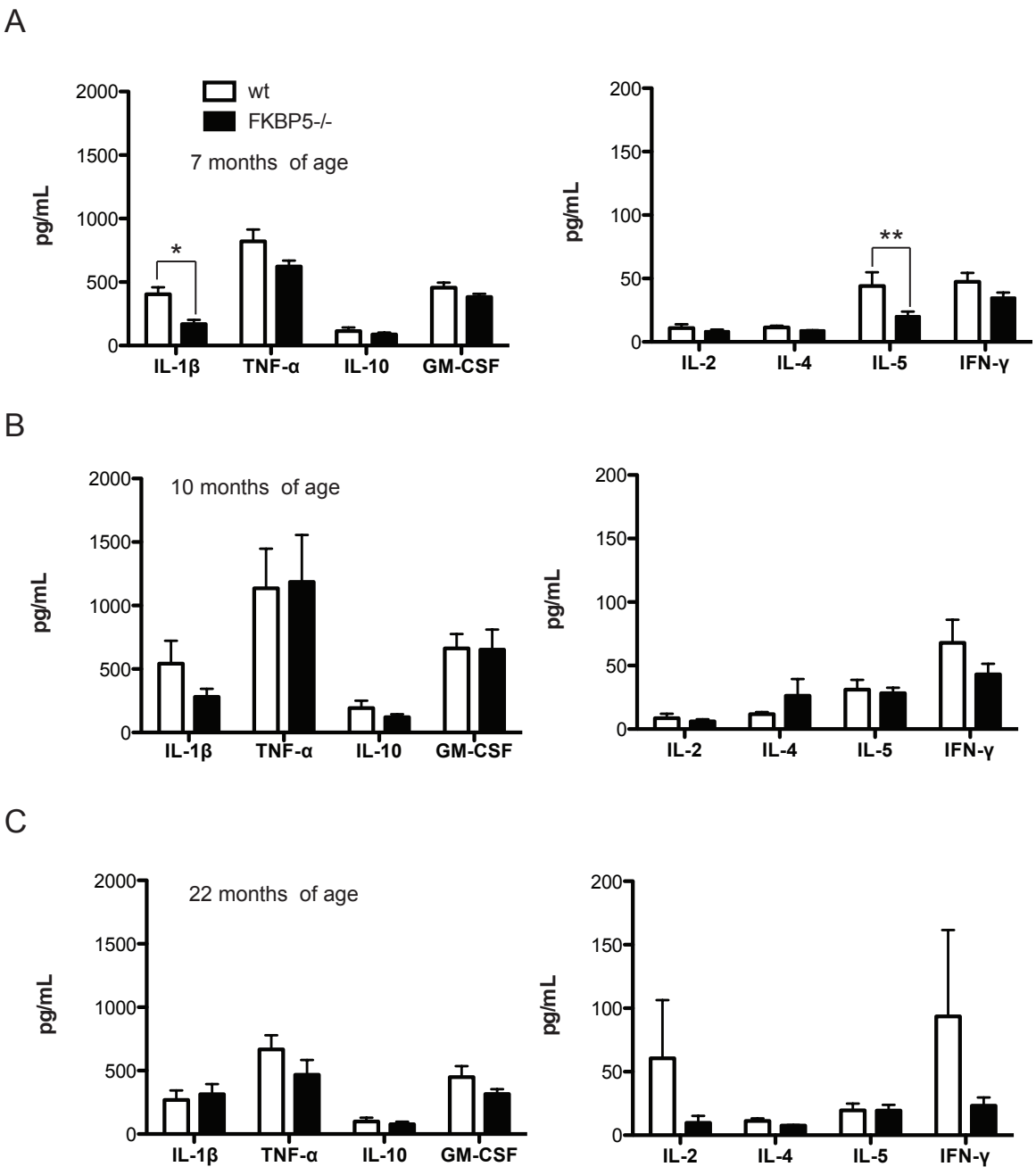

A
wt
FKBP5-/-
7 months of age
pg/mL
2000
1500
1000
500
0
*
IL-1β
TNF-α
IL-10
GM-CSF
200
150
100
50
0
**
IL-2
IL-4
IL-5
IFN-γ
B
10 months of age
C
22 months of age

Figure 4.5

Figure 4.4. IL-1β and IL-5 are reduced in the FKBP5-/- mice at 7 months of age. (A) FKBP5-/- mice of 7 months of age display reduced levels of basal serum IL-1β, and IL-5 as compared to wild type mice. (B) FKBP5-/- mice do not show statistical difference in the levels of basal serum cytokines at 10 months of age. (C) FKBP5-/- mice do not show a statistical difference in the levels of basal serum cytokines at 22 months of age. *$p<0.05$, **$p<0.01$.

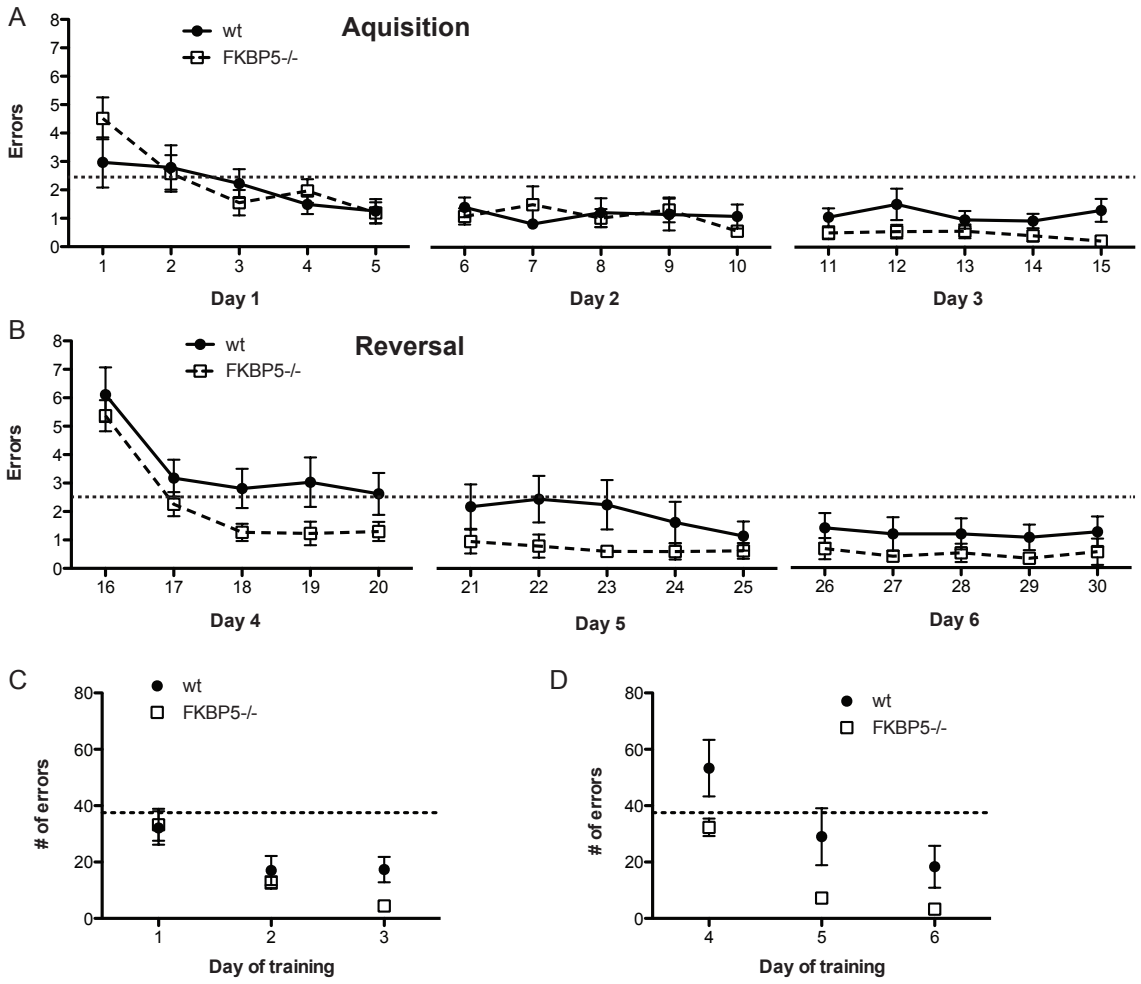
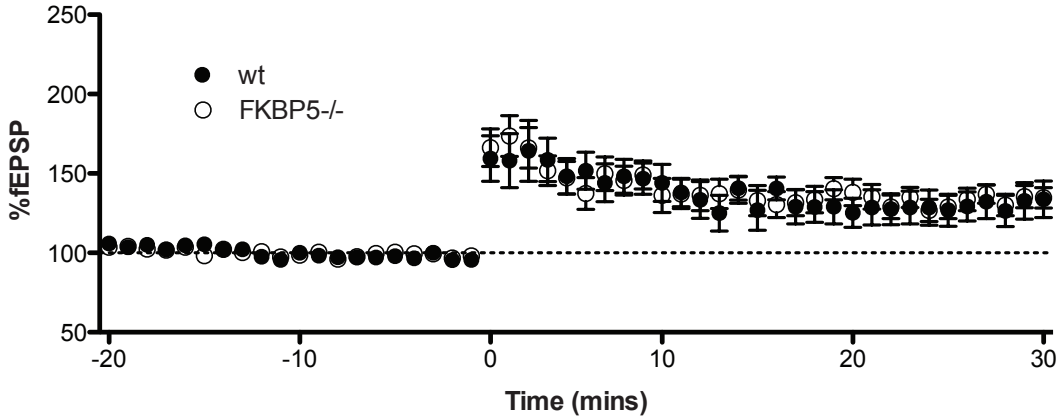

A
Aquisition
wt
FKBP5-/-
Errors
Day 1
Day 2
Day 3
B
Reversal
wt
FKBP5-/-
Errors
Day 4
Day 5
Day 6
C
wt
FKBP5-/-
# of errors
Day of training
D
wt
FKBP5-/-
# of errors
Day of training

Figure 4.5

Figure 4.5. FKBP5 ablation increases cognitive flexibility. (A) Acquisition phase of the six radial arm water maze. Each number is a group of three trials. (B) Reversal of the location of the hidden platform. (C) Total number of errors per day in the initial acquisition phase of learning the location of the escape platform. (D) Total number of errors per day in the reversal of the escape platform ($F(1,16)=4.9$, $*p=0.0417$). Dashed line represents the probability of the number of errors per day by chance. Chance is accepted as 2.5 errors per trial, and there are 15 trials per day.

A


250
200
150
100
50
%fEPSP
wt
FKBP5-/-
-20
-10
0
10
20
30
Time (mins)

B

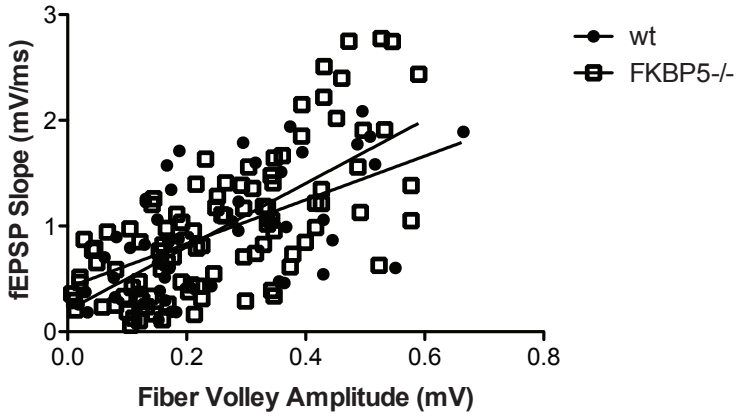
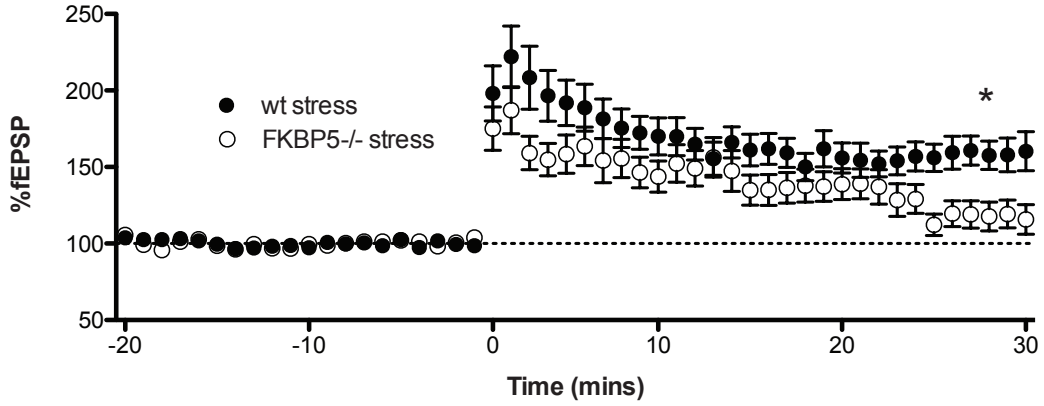

3
2
1
0
fEPSP Slope (mV/ms)
0.0
0.2
0.4
0.6
0.8
Fiber Volley Amplitude (mV)
wt
FKBP5-/-

Figure 4.6

Figure 4.6. Knockout of FKBP5 gene does not affect long term potentiation. (A) 30 minutes of long term potentiation does not show a deficit between wild type and FKBP5-/- mice. (B) Equal presynaptic stimulus does not produce differential postsynaptic response.

A


250
200
150
100
50
%fEPSP
wt stress
FKBP5-/- stress
*
-20
-10
0
10
20
30
Time (mins)

B

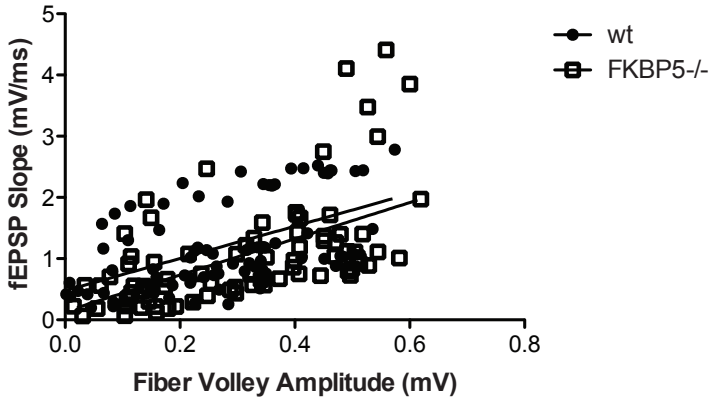

5
4
3
2
1
0
fEPSP Slope (mV/ms)
0.0
0.2
0.4
0.6
0.8
Fiber Volley Amplitude (mV)
wt
FKBP5-/-

Figure 4.7

Figure 4.7. Long-term potentiation is enhanced in wild type mice after acute restraint stress, but not in FKBP5-/- mice. (A) Hippocampal long-term potentiation of mice after 1 hour of acute restraint stress displays FKBP5-/- mice do not potentiate or maintain LTP to the extent of wild type mice. (B) Equal presynaptic stimulus does not produce differential postsynaptic response.

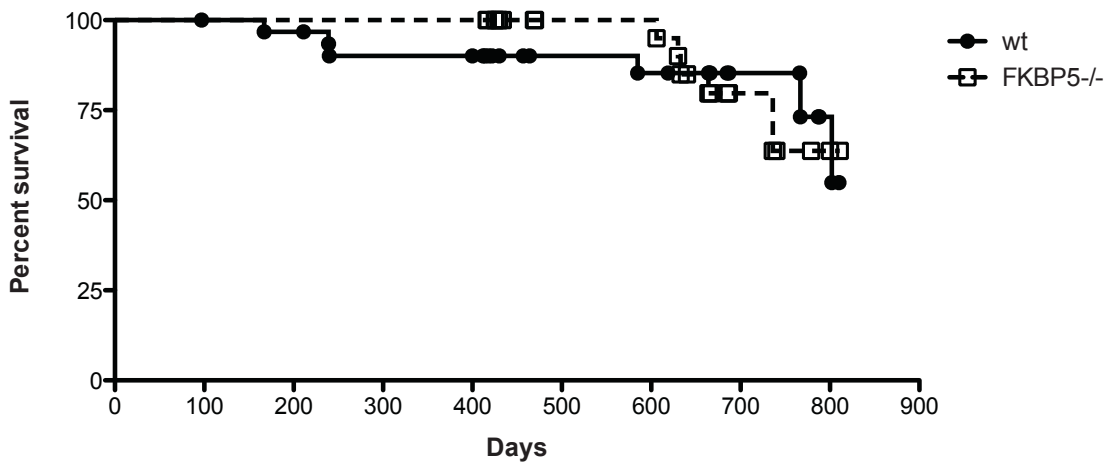

Percent survival
100
75
50
25
0
0
100
200
300
400
500
600
700
800
900
Days
wt
FKBP5-/-

Figure 4.8

Figure 4.8. Ablation of the *FKBP5* gene does not alter lifespan. Survival curve of wild type and FKBP5-/- mice. Approximately 50% of both cohorts are still alive at 800 days (χ^2= 0.0014, p=0.9707).

CHAPTER FIVE:

Discussion & Conclusions

Hsp70 and Methylene Blue

In the introduction we outlined that the targeting of soluble tau is a promising therapeutic avenue for the treatment of AD. In "Chapter Two" we tested this hypothesis with methylene blue, due to its ability to inhibit Hsp70 ATPase activity and reduce tau levels in various neuronal and non-neuronal cell models. Methylene blue was able to reduce tau when found in large concentrations in the brain. It was also able to rescue neuronal death. Interestingly, cognition was only restored in animals where tau levels were lowered. Mice with low levels of methylene blue displayed robust neuroprotection, but no reduction in tau. As a result, there was no improvement in cognition, suggesting that protection from tau-induced neuronal malfunction came from lessening the tau burden. Keeping more neurons alive was only beneficial when tau was reduced. This indicates that keeping

neurons alive is not sufficient for the treatment of AD. Removing tau, however, allows neurons to communicate properly to establish function, in this case memory. This idea is further discussed in Appendix C.

Methylene blue is a tau fibril inhibitor?

Despite its success in clinical trials and in animal models, there is some controversy as to the mechanism(s) of methylene blue. *In vitro*, methylene blue displays an inhibition of tau fibrillization, potentially through the oxidation of cysteine residues (Wischik et al., 1996; Miyata et al., 2012; Akoury et al., 2013). However, there is yet to be a study where methylene blue treatment retards, prevents or reduces tau tangles *in vivo*, including the study in this dissertation, "Chapter Two." Here, there were no differences in tangle load in mice treated with methylene blue in their water at 165 μM, or direct injection to the brain at 1 mM concentration. But as has been previously established, a change in tangle load may not be necessary to improve cognition. Others have also found methylene blue treatment to be beneficial in other AD, tau, and other neurodegenerative mouse models (Deiana et al., 2009; Medina et al., 2010; Congdon et al., 2012; Hosokawa et al., 2012).

Future work

Although we do not know the precise mechanism of how methylene blue affects tau, it is important to note that it has shown positive effects in phase 2 clinical trials (Gura, 2008; Wischik and Staff, 2009). TauRx®, the company that put methylene blue through the trials, has patented an analog of the compound called leuco-methylthioninium, LMTX™, and has begun enrolling patients into phase three clinical trials (Yan, 2012). This is the most exciting part, to think that soon a new therapeutic option may be available. However, further work into the Hsp70 chaperone network has revealed that different Hsp70 proteins have differential effects on tau levels (Jinwal et al., 2013). In fact, there are 11 human Hsp70 genes, according to one article (Tavaria et al., 1996). However, an extensive discussion on these genes argues that 5 of these are not real, and thus there are really 7 Hsp70 genes that make a protein (Moran). The *HSPA1L* gene is specific to the testis, the *HSPA5* is specific to the ER, and the *HSPA9B* is specific to the mitochondria. The rest are cytosolic, the *HSPA1A* and he *HSPA1B*, are inducible, and *HSPA2* and *HSPA8*, are constitutively expressed.The work of Dr. Chad Dickey and Dr. Umesh Jinwal suggest that there is a chaperone "code," which helps create proteostasis in the cell (Jinwal et al., 2010; Jinwal et al., 2011; Jinwal

et al., 2013). Future work must elucidate which genes are good for tau degradation and which ones promote tau accumulation. Then, we have to develop strategies to fine tune the activities of these genes to promote tau clearance.

Implications

This study on methylene blue goes beyond the ability of methylene blue to affect cognition in Alzheimer's disease. It is part of a fuselage of work from Dr. Dickey's laboratory showing the potential that chaperone proteins have as targets from which therapies can be developed for the treatment of neurodegenerative diseases. This study is also important for the tau field, as these data support the previous work showing that reducing the levels of soluble tau is beneficial (Santacruz et al., 2005) (de Calignon et al., 2010) . The reduction of the tau burden in these mice was not detrimental to their locomotion or motor skills, as suggested it would be by the study showing tau knockout mice having motor issues with age. On the contrary, mice regained cognitive function from tau removal. Tau tangles were not removed and the number of tangles remained steady, and the result was not affected by this.

FKBP5 and Stress Disorders

FKBP5 levels are elevated in people with SNPs in the *FKBP5* gene. These people are overrepresented in the population of those who have experienced severe trauma and develop mood disorders. In "Chapter Three" and "Chapter Four," we tested the hypothesis that the absence of FKBP5 would protect from developing disease. The FKBP5-/- mice were protected from an increase in age-related corticosterone levels, and displayed increased resilience and cognitive flexibility, both due to stress and age. Natural resilience decreased with age in wild type mice, but the FKBP5-/- mice were protected from this. Moreover, global knockout of this gene was not deleterious in any measurements done in these studies.

Future work

Several experiments need to be done to further validate the results in this dissertation. First, we performed these experiments on a permanent knockout mouse line. Accordingly, we need to test if the transient knockout of this gene is as effective in treating disease. Moreover, this mouse line is a global knockout, thus the transient knockout mouse should be a brain only knockout as well. After this validation, therapeutic strategies need to be developed to reduce the

levels of FKBP5 in the brain, and then translated into human clinical trials.

Implications

There has been a lack of development of new therapies in the realm of mood and anxiety disorders from the use of hypothesis driven science (2007). As a result, these studies are very exciting, as we have a good general understanding of the mechanism of how the environment is changing our genetic regulation, thus making us more susceptible to disease. Traditionally, the ideas are first developed in animal models and then tested in humans. Here, it has been the opposite. Since FKBP5 is ubiquitous in the human body, mRNA levels can be tested in blood cells, and these highly correlate with brain mRNA levels (Sullivan et al., 2006). As a result, we know that chronic elevation of FKBP5 levels is bad for people. Armed with this knowledge we can develop therapies aimed at decreasing the levels of FKBP5.

Very little work has been done with resilience and the genetic causes of resilience, but even less on the resilience of the elderly. This work shows that the absence of FKBP5 provides mice with an enhancement in resilience that extends on to old age. There is also a very strong correlation between elevated cortisol levels and psychiatric disease (Pariante and Miller, 2001). As a result, the reduced cortisol

phenotype observed in these mice has the potential to alleviate the risk of developing different types of mood and anxiety disorders.

Conclusion

Chaperone proteins are very important in the cell. They are responsible for folding, transporting, receptor function, assisting endocytic vesicles, decreasing levels of reactive oxygen species, vesicle fusion, protein degradation, preventing protein aggregation, and ER associated degradation, among others (Muchowski and Wacker, 2005). Despite this, their function is not always sufficient to prevent disease. Because of their multiple talents, though, we can modulate them to restore health, and prevent disease.

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Jinwal UK, Trotter JH, Abisambra JF, Koren J, 3rd, Lawson LY, Vestal GD, O'Leary JC, 3rd, Johnson AG, Jin Y, Jones JR, Li Q, Weeber EJ, Dickey CA (2011) The Hsp90 kinase co-chaperone Cdc37 regulates tau stability and phosphorylation dynamics. J Biol Chem 286:16976-16983.

Jinwal UK, Koren J, 3rd, Borysov SI, Schmid AB, Abisambra JF, Blair LJ, Johnson AG, Jones JR, Shults CL, O'Leary JC, 3rd, Jin Y, Buchner J, Cox MB, Dickey CA (2010) The Hsp90 cochaperone, FKBP51, increases Tau stability and polymerizes microtubules. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:591-599.

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APPENDIX A:

Chapter Two Supplementary Material

Appendix A consists of the supplementary material from "Chapter 2: Phenothiazine-mediated rescue of cognition in tau transgenic mice requires neuroprotection and reduced soluble tau burden."

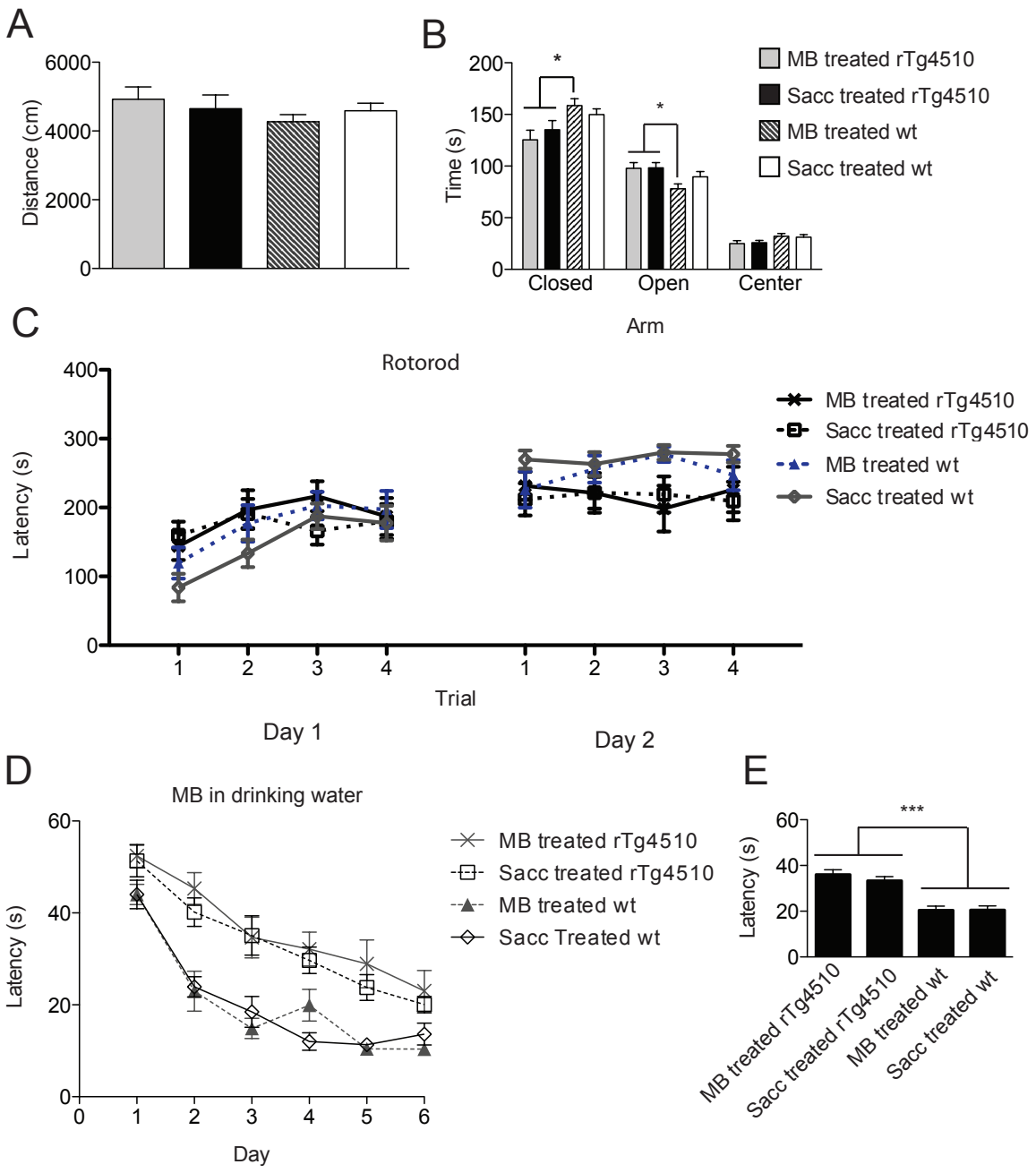

A
Distance (cm)
6000
4000
2000
0
B
Time (s)
200
150
100
50
0
*
*
Closed
Open
Center
Arm
MB treated rTg4510
Sacc treated rTg4510
MB treated wt
Sacc treated wt
C
Rotorod
Latency (s)
400
300
200
100
0
1
2
3
4
Trial
Day 1
Day 2
D
MB in drinking water
Latency (s)
60
40
20
0
0
1
2
3
4
5
6
Day
MB treated rTg4510
Sacc treated rTg4510
MB treated wt
Sacc Treated wt
E
Latency (s)
60
40
20
0
***
MB treated rTg4510
Sacc treated rTg4510
MB treated wt
Sacc treated wt

Figure A1

Figure A1. Chronic MB treatment does not cause abnormal changes in mice behavior. (A) Mice were assessed in the open field task for abnormal locomotion and exploratory behavior for 30 minutes. No statistical differences were observed. (B) Mice were evaluated for anxiety in the elevated plus maze task. The amount of time spent in the open and closed arms, and the center area was evaluated and at least one mean was indicated to be significant ($F(3,36) = 3.414$, $p<0.05$). Post-hoc evaluation shows that MB treated wild type mice displayed significantly more time in the closed arms and significantly less time in the open arms than both cohorts of transgenic mice. (C) Mice were subjected to the rotorod apparatus for 4 trials a day for 2 days. No overt differences were observed. (D) Mice positive for human P301L tau transgene expression and their wild type littermates were grouped based on genotype and MB treatment. On the 14th week of chronic MB treatment they were subjected to the water maze paradigm. Learning was evaluated by the latency of the mice to find the hidden platform. The mice were trained until the wild type mice plateaued. Wild Type animals learned significantly better than transgenic animals ($n=10$ per group, $F(3, 236) = 19.71$, $p<0.0001$). (E) Graphic representation of the mean latency of all mice in all trials of plot d.

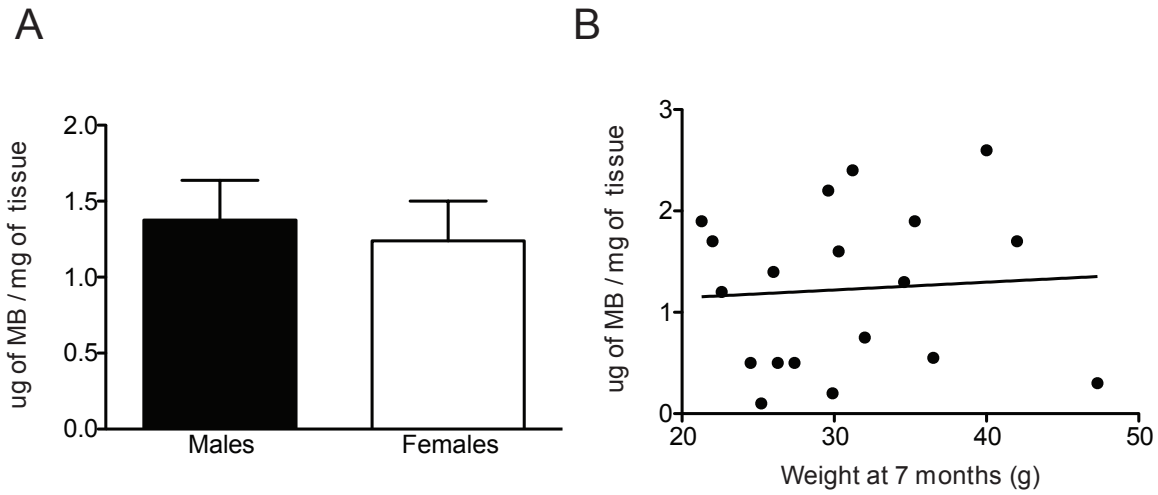
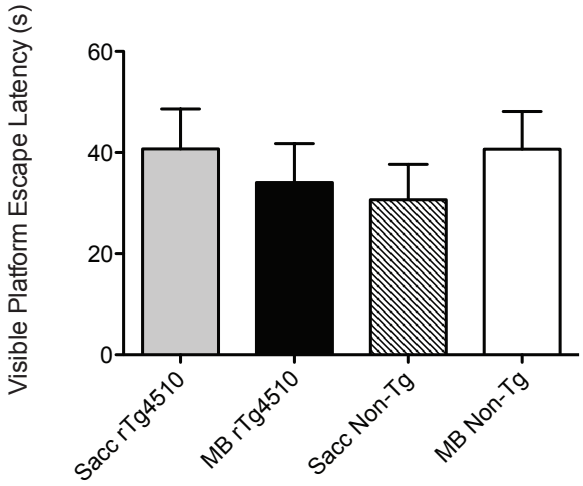

A
B
ug of MB / mg of tissue
2.0
1.5
1.0
0.5
0.0
Males
Females
ug of MB / mg of tissue
3
2
1
0
20
30
40
50
Weight at 7 months (g)

Figure A2

Figure A2. Differences in parenchymal drug concentrations could not be attributed to gender or weight. (A) All drug treated mice (both transgenic and wild type) separated by gender show no statistical difference between the means, p=0.7197 (males n=10, females n=9). (B) Linear regression and correlation between weight and drug concentration show that slope is not significantly non-zero and correlation is not statistically significant, p=0.7778 (n=19).

A


Visible Platform Escape Latency (s)
60
40
20
0
Sacc rTg4510
MB rTg4510
Sacc Non-Tg
MB Non-Tg

B

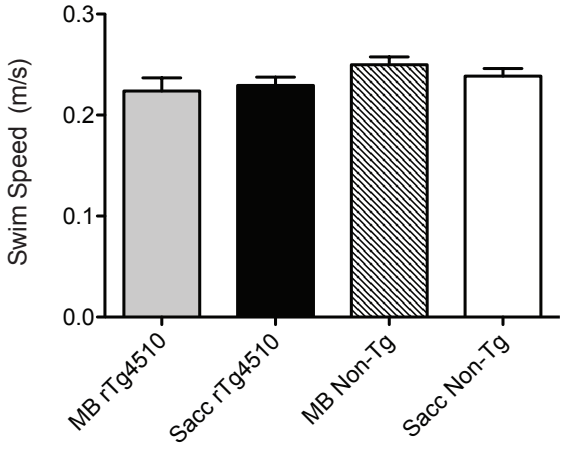

Swim Speed (m/s)
0.3
0.2
0.1
0.0
MB rTg4510
Sacc rTg4510
MB Non-Tg
Sacc Non-Tg

Figure A3

Figure A3. Mice treated with methylene blue display normal vision and swim speed. (A) Visible trial in the Morris water maze displays no significant difference between groups. (B) Swim speed in the Morris water maze shows no significant difference between the groups.

APPENDIX B[3]:

Hsc70 Rapidly Engages Tau after Microtubule Destabilization

Abstract

The microtubule-associated protein Tau plays a crucial role in regulating the dynamic stability of microtubules during neuronal development and synaptic transmission. In a group of neurodegenerative diseases, such as Alzheimer disease and other tauopathies, conformational changes in Tau are associated with the initial stages of disease pathology. Folding of Tau into the MC1 conformation, where the amino acids at residues 7–9 interact with residues 312–342, is one of the earliest pathological alterations of Tau in Alzheimer disease. The mechanism of this conformational change in

[3]This work was previously published in the *Journal of Biological Chemistry* (Jinwal, U. K., O'Leary, J. C., Borysov, S. I., Jones, J. R., Li, Q., Koren, J., ... & Dickey, C. A. (2010). Hsc70 rapidly engages tau after microtubule destabilization. Journal of Biological Chemistry, 285(22), 16798-16805.), and is used here with permission of the publisher. The permissions are available in Appendix F. © the American Society for Biochemistry and Molecular Biology.

Tau and the subsequent effect on function and association to microtubules is largely unknown. Recent work by our group and others suggests that members of the Hsp70 family play a significant role in Tau regulation. Our new findings suggest that heat shock cognate (Hsc) 70 facilitates Tau-mediated microtubule polymerization. The association of Hsc70 with Tau was rapidly enhanced following treatment with microtubule-destabilizing agents. The fate of Tau released from the microtubule was found to be dependent on ATPase activity of Hsc70. Microtubule destabilization also rapidly increased the MC1 folded conformation of Tau. An in vitro assay suggests that Hsc70 facilitates formation of MC1 Tau. However, in a hyperphosphorylating environment, the formation of MC1 was abrogated, but Hsc70 binding to Tau was enhanced. Thus, under normal circumstances, MC1 formation may be a protective conformation facilitated by Hsc70. However, in a diseased environment, Hsc70 may preserve Tau in a more unstructured state, perhaps facilitating its pathogenicity.

Introduction

Deposition of Tau after its sequestration from microtubules is a neuropathological hallmark of a group of diseases termed tauopathies. This family of diseases includes Alzheimer disease (AD),3 hereditary frontotemporal dementia with parkinsonism linked to chromosome 17, progressive supranuclear palsy, and corticobasal degeneration (1,–4). The Tau protein is normally expressed in the cytoplasm of neurons and performs the critical function of stabilizing and maintaining the microtubule networks that are essential for axonal transport. During the development of Tau pathology, Tau dissociates from microtubules, and unknown factors converge to cause abnormal polymerization of Tau into highly insoluble paired helical filaments (PHF) and forms neurofibillary tangles. The number of neurofibillary tangles directly correlates with the degree of dementia seen in the progressing stages of AD (5, 6); however, it is now speculated that it is intermediate species of Tau rather than the pathologically visible tangles that are the most toxic (7, 8). It has been hypothesized that in disease, hyperphosphorylation and conformational changes lead to the loss of function and pathogenic assembly of Tau (9,–11). This idea of Tau adopting a conformation, or “fold,” is somewhat paradoxical because Tau is almost entirely an unstructured protein. However, evidence for a

folded Tau species emerged from studies that used paired helical filaments from AD brain as haptens for monoclonal antibody production (10,–12). Several antibodies were generated that had discontinuous epitopes within the Tau molecule. One of these antibodies, MC1, recognizes Tau when its N terminus is positioned near residues of the ninth exon. Pathological studies with this antibody revealed that formation of this species was an early event in Tau pathology in AD (10,–12). Thus, detection of Tau in the MC1 conformation could result from either folding of a single Tau molecule, or it could be due to anti-parallel stacking/filament formation of Tau.

The emergence of molecular chaperones as key regulators of Tau processing suggests that conformational changes to the structure of Tau may indeed be key events in the pathogenesis of AD and other tauopathies (13,–17). Aggregation of Tau in vitro is negligible and typically requires accelerants such as heparin (18, 19), an indication that conformation plays an important role in its assembly. In a cellular environment, post-translational processing of Tau is likely essential for facilitating its aggregation, and certainly this could be regulated by the chaperone network. One of the first described chaperone regulators of Tau is the Hsp70 chaperone family. This family is comprised of 13 members; however, the cytoplasm is predominated by two members, the constitutive heat shock cognate (Hsc) 70 protein and the inducible

Hsp70. Hsc70 and Hsp70 share 92% primary sequence homology. Both have highly conserved N-terminal ATPase domains as well as substrate-binding domains located just upstream of a more variable/ regulatory domain (20). The cyto-protective role of Hsc70 may be particularly relevant in neurons where an attenuated Hsp70 response has been correlated with aging. Hsc70 and Hsp70 can each bind Tau in the nervous system, but endogenous Hsc70 is typically in much higher abundance than Hsp70 in the cytosol (21, 22). Based on previous work, we hypothesized that the highly abundant Hsc70 protein would be the most likely member of the Hsp70 family to regulate Tau processing. We also speculated that the time when Hsc70 would be most engaged with Tau would be following its release from microtubules (17). Lastly, we investigated what affect Hsc70 might have on Tau in distinct cellular environments. For the first time, we have connected microtubule dysfunction with chaperone-mediated regulation of Tau, as well as conformational changes to and hyperphosphorylation of Tau in a comprehensive way. These findings could be useful for implementing therapeutic strategies based on Hsc70, given its apparent early involvement in Tau pathogenicity. These studies may also point to a mechanism of how Tau dysfunction begins in AD and other tauopathies.

Materials & Methods

Antibodies and chemicals

MC1 and PHF1 (anti-S396/S404 p-Tau) were provided by P. Davies (Albert Einstein College of Medicine, Yeshiva University, New York, NY). Anti-Hsc70 was obtained from Stressgen Biotechnologies (Ann Arbor, MI). Anti-actin was obtained from Sigma-Aldrich. Anti-Tau (total Tau) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Secondary antibodies were obtained from Southern Biotech (Birmingham, AL). All of the antibodies were used at a 1:1,000 dilution with the exception of PHF1, which was used at a dilution of 1:200. Secondary antibodies conjugated to fluorophore were purchased from AlexaFluor (Molecular Probes). Albendazole, colchicine, paclitaxel, okadaic acid, and phenothiazine (methylene blue) were purchased from Sigma. Nocodazole was purchased from Tocris Bioscience.

Recombinant protein purification

Wild type 4R Tau and Hsc70 gene sequences cloned into pET28 vector with His tag and were transformed into the Escherichia coli strain BL21 (DE3) codon Plus. Bacterial culture condition and protein purification protocol were followed as described earlier (23). The purity

of all proteins was verified on a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel.

Xenopus oocytes

S phase Xenopus egg extracts were prepared according to a standard protocol (24) and supplemented with 5% Me2SO to stimulate microtubule polymerization as described (23). Purified recombinant wild type Tau and Hsc70 proteins were added at a final concentration of 80 ng/µl. The extracts were treated with nocodazole and incubated for 30 min at room temperature, and newly formed microtubule structures were visualized by fluorescence of incorporated rhodamine-tubuline. Image quantification was performed as previously described (25). Briefly, tubulin-positive signal was detected using the same threshold for each image in ImageJ (26, 27), and the fraction of the total area of the field covered by the tubulin signal was collected for statistical analysis.

Cell culture and western blot analysis

HeLa cells stably transfected with V5-tagged 4R0N Tau were generated using G418 selection. HeLa cells were grown in Opti-Mem plus 10% fetal bovine serum (complete media; Invitrogen) and passaged every 3–5 days based on 90% confluence. IMR32 cells were

maintained in Opti-Mem plus 10% fetal bovine serum and 2% of 200 mm l-glutamine (Cellgro, Mediatech, Inc., Herndon, VA). The cells were harvested in M-PER buffer (Pierce) containing 1× protease inhibitor mixture (Calbiochem), 1 mm phenylmethylsulfonyl fluoride, and 1× phosphatase inhibitor I and II cocktails (Sigma). The measurements of Tau levels in cell culture were performed by Western blot analysis. Plasmid transfections were done utilizing Lipofectamine 2000 reagent from Invitrogen. HeLa cells stably transfected with V5-tagged 4R human Tau were transfected with 3 μg of DNA. The cells were incubated for 4 h with the Lipofectamine/plasmid mixture in Opti-MEM medium, and this was replaced with fresh complete medium for an additional 44–48 h. For the immunoprecipitation (IP) studies, cell culture lysates were incubated with 2 μg of antibody for 2–3 h at 4 °C with rocking. Then 50 μl of protein G were added and rocked at 4 °C for overnight. The protein G beads were pelleted and washed five times with phosphate-buffered saline.

Drug preparations

All of the drugs for the cell culture experiments were solubilized in Me2SO to get a 20 mm stock. For the in vivo study in mice, albendazole used in the hippocampal injections in Figure B3 panel C

were prepared by dissolving 3 g of albendazole in 40% hydroxypropyl-β-cyclodextrin in water.

Immunocytochemistry

IMR32 cells were plated onto a chamber slide (Fisher) and maintained in the medium described above. The cells were treated with nocodazole for 30 min and were fixed in high pressure liquid chromatography grade methanol for 15 min. Blocking and permeabilization were done by incubating samples with Tris-buffered saline containing 5% normal goat serum, 0.1% Tween 20, and 0.03 NaN3 for 60 min at 25 °C. Primary antibodies rabbit anti-Tau and rat anti-Hsc70 (1:100) were added and incubated overnight at 4 °C. The slide was washed five times for 5 min in 1× Tris-buffered saline. Secondary antibodies AlexaFluor 488 (anti-rabbit; 1:1000) and 594 (anti-rat; 1:1000) were added to the slides and incubated for 60 min at 25 °C. The slides were washed and coverslipped. A Leica confocal microscope was used to take images. Significance was assessed by Pearson's correlation coefficient.

Albendazole injections in mouse brains

All of the procedures using mice were done in accord with the guidelines set forth by the University of South Florida's Institutional

Animal Care and Use Committee. The wild type FVB/N background mice brain hippocampus were injected with 2 µl of vehicle and 3 mg/ml albendazole. The surgical procedure was performed as previously described by Carty et al. (28) with some modifications. The mice were anesthetized using isofluorane, and the cranium was exposed with a small incision along the skin covering the medial sagittal plane. Holes were drilled through the cranium over the desired injection sites as measured with a stereotaxic apparatus. Burr holes were drilled using a dental drill bit (SSW HP-3; SSWhiteBurs) or a 21-gauge needle (BD). A 2-µl total volume of vehicle or albendazole were dispensed into the each injection site. The animals were recovered within 10 min of post-surgery and were singly housed. 24 h after the injections, the brains were harvested as described earlier (29) and processed for immunoprecipitation as described above.

Enzyme-linked immunosorbent assay

Tau and HSP complex containing Hsc70, DNAJB2, and ATP were preincubated for 30 min. Paraformaldehyde was added into the samples and coated on to 96-well plate for 1 h at 37 °C followed by washing five times with wash buffer containing 0.45% NaCl and 0.05% Tween 20. Blocking of plate was done for 1 h at 37 °C by using starting blocking solution (Pierce and Thermo Fisher Scientific),

followed by a wash. MC1 antibody was added at 1:50 dilutions and incubated at 37 °C for 1 h, and the plate was washed again. Horseradish peroxidase-linked secondary antibody was added and incubated at 37 °C for 1 h. The plate was washed. 3,3′,5,5′-Tetramethylbenzidine dihydrochloride (Sigma) solution was added, and the reaction was stopped by adding 2 m H2SO4. The plate was read at 450 nm in spectrophotometer.

Results

Hsc70 facilitates tau-mediated microtubule stabilization

Previous work from our lab and others suggested that the Hsp70 chaperone network plays a critical role in Tau biology and could be used as a therapeutic target for tauopathies (13, 14, 17, 30, 31). Of the Hsp70 family members, the Hsc70 protein expression level was found to be highest in the neurons from adult brain (21, 32,–34). Based on this evidence, we investigated the role of Hsc70 on Tau-mediated microtubule polymerization. We used our recently optimized assay for the analysis of microtubule polymerization in oocyte extract (23). Using purified recombinant Hsc70 and Tau, we incubated Xenopus oocyte extracts with Tau, Hsc70, or a combination of both Tau and Hsc70 for 30 min. These extracts were then treated with 20 μm

nocodazole or vehicle for an additional 30 min. Microtubule formation was analyzed using fluorescent microscopy (Figure B1). Tubulin-positive signal was detected using the same threshold for each image as determined by ImageJ software (26, 27), and the fraction of the total area of the field covered by the tubulin signal was collected for statistical analysis (Figure B1 B). The extracts incubated with Tau alone and Hsc70 alone showed similar levels of microtubule formation (Figure B1). However, we observed a significant increase in microtubule formation when Tau was supplemented with Hsc70. Nocodazole was able to abrogate microtubule formation under all the previously mentioned conditions as seen in the representative image (Figure B1 A). These findings suggest that Hsc70 could be a potent enhancer of Tau-mediated microtubule polymerization, suggesting that microtubule dynamics could be critical for facilitating the Tau/Hsc70 interface.

Tau associates with Hsc70 upon microtubule destabilization

Although Hsc70 facilitated Tau-mediated microtubule assembly in normal cellular oocyte extracts, we found that microtubule destabilization with nocodazole overrode this activity (Figure B1). Based on this result, we wanted to determine how microtubule destabilization may impact the association of Hsc70 with endogenous

Tau in an intact cellular environment. First, we performed fluorescent immunostaining on neuroblastoma cells (IMR32) following microtubule destabilization to determine whether colocalization of these two proteins was increased. The cells were grown on chamber slides and treated with nocodazole for 30 min and then fixed, and the levels of Tau and Hsc70 were assessed by immunofluorescent confocal microscopy. Image analysis showed no significant colocalization between Tau and Hsc70 in the vehicle-treated cells as determined by Pearson's correlation coefficient; however, Hsc70 and Tau were found to be colocalized by Pearson's correlation coefficient after nocodazole treatment (Figure B2). These data suggested that Hsc70 and Tau do have a greater propensity to interact following microtubule destabilization. However, we wanted to further confirm these data using coimmunoprecipitation studies.

We tested whether the interaction of Tau with Hsc70 could be enhanced with microtubule destabilization in cells overexpressing Tau, as well as cells with endogenous Tau levels. We treated IMR32 cells that have endogenous levels of Tau with three microtubule-destabilizing drugs: albendazole, nocodazole, and colchicine. These compounds did not increase Hsc70 levels; however, consistent with our colocalization data, they did increase the binding of Tau with HSc70

(Figure B3, A and B). A similar result was found with HeLa cell overexpressing wild type human Tau (Figure B3 C). As a control in this overexpressing cell model, we also treated with the microtubule stabilizer, paclitaxel, and saw no increase in Tau association to Hsc70 (Figure B3 C). We then tested whether the association of Hsc70 with Tau could be increased by microtubule destabilization in the brain. We injected albendazole and vehicle control into the hippocampal region of mice. Brain tissue was harvested 24 h after injection, and homogenates were analyzed by an IP with Tau antibody followed by Western blot probing with Hsc70 antibody. Again, we found that microtubule destabilization increased the association of Tau with Hsc70 (Figure B3 D).

ATPase activity of Hsp70/Hsc70 regulates tau upon microtubule destabilization

Based on these findings and our previous work with Hsp/Hsc70 chemical inhibitors (14), we speculated that chemically inhibiting Hsc70 activity when the number of Hsc70·Tau complexes is highest (after microtubule destabilization) would lead to enhanced efficacy for reducing Tau levels. To determine this, we performed dose-response studies with an Hsp70 ATPase inhibitor (methylene blue) in the presence or absence of a 30-min pretreatment with albendazole.

Indeed we found that reductions in both endogenous and overexpressed Tau by Hsc70 inhibition were enhanced following microtubule destabilization in IMR32 cells and HeLa cells, respectively (Figure B4). These findings provide further evidence that the association of Hsc70 with Tau is rapidly enhanced following microtubule destabilization, which forms more Hsc70·Tau complexes that can be targeted by inhibitors.

Hsc70 folds tau into MC1 conformation

Based on our results, we hypothesized that Hsc70 could be contributing to some of the early pathological modifications to Tau depending on the cellular environment. Because Hsc70 largely functions as a protein "foldase" (35), and Tau folding is thought to be an early event in disease progression (11, 12, 36), we investigated what impact Hsc70 could be having on Tau folding.

We first tested whether the folding of Tau into the MC1 conformation coincided with Hsc70 binding to Tau. Again we treated two cell models, one with endogenous Tau and the other with overexpressed Tau, with microtubule destabilizers for 30 min and performed IPs with the MC1 antibody, which recognizes folded Tau, and a total Tau antibody. We found that MC1-reactive Tau was

increased within 30 min in both cell models, similar to the time frame of Hsc70 binding to Tau (Figure B5, A and B); however, this effect was more pronounced in cells overexpressing Tau (Figure B5 B). Although this suggested that Hsc70 and Tau folding were occurring in a similar time frame, we still were not certain that Hsc70 was facilitating the MC1 conformation for Tau. To test this more directly, we performed an in vitro enzyme-linked immunosorbent assay experiment using purified recombinant proteins to look for the amount of MC1 reactive Tau in the presence and absence of Hsc70. Impressively, we found more MC1-reactive Tau species when Tau was incubated with Hsc70 versus bovine serum albumin (Figure B5 C). Based on our findings that the MC1 Tau conformation could be rapidly facilitated by Hsc70, we speculated that Tau hyperphosphorylation might be impacted as well when microtubules are destabilized.

Microtubule destabilization affects tau phosphorylation pattern

We first investigated the phosphorylation of Tau within the first 24 h following microtubule destabilization. Again we investigated this for both endogenous Tau and overexpressed Tau. Interestingly, we observed an initial decrease in Tau phosphorylation at 30 min, the same time that MC1 Tau formation and Hsc70 binding to Tau were increased. Tau phosphorylation then began to recover over the next

several hours and eventually was increased by 24 h in both cell lines (Figure B6). Total Tau levels remained largely unaffected. Thus, microtubule destabilization decouples Tau phosphorylation from MC1 formation and Hsc70 binding. Perhaps more importantly, MC1 formation and Hsc70 actually preceded endogenous phosphorylation of Tau following microtubule destabilization. This led us to consider what would happen to both the MC1 conformation of Tau and Hsc70 binding to Tau if microtubules were destabilized in the face of aberrant kinase activation.

A hyperphosphorylating environment blocks folding of tau but enhances Hsc70 binding

Although we have demonstrated that Hsc70 associates to Tau upon microtubule destabilization, we wanted to determine how this association is affected in a hyperphosphorylating environment. Tau protein can be phosphorylated by a number of proline-directed serine/ threonine kinases at several sites. The prevalence and chronology of these phosphorylation events have been rigorously correlated with AD tangle pathology (5, 6). GSK3β is a primary kinase for several key sites that are associated with Tau pathology, and it can lead to Tau hyperphosphorylation (37). Here, we have created a hyperphosphorylating environment for Tau by overexpressing a

constitutively active form of GSK3β. Lysates from cells overexpressing either the constitutively active form of GSK3β or empty vector treated with nocodazole for 30 min were analyzed by IP as described above. Although we found that phosphorylation of Tau (as measured by increased immunoreactivity to PHF1 antibody) did increase its association with Hsc70, we were surprised to find that hyperphosphorylation of Tau actually blocked MC1 formation (Figure B7). Thus, when microtubule destabilization occurs in a hyperphosphorylating environment, MC1 formation is blocked, but Hsc70 binding goes up. In this way, Hsc70 may be preserving hyperphosphorylated Tau in a more exposed, linear conformation that may be more prone to toxic intracellular interactions than it would be in the condensed MC1 conformation.

Discussion

Apart from the known association of the members of the Hsp70 family of chaperones with Tau in the brain, their role in the initiation and progression of AD and other tauopathies is not clear. Here, we aimed to explore the role of the most prevalent cytosolic member of the Hsp70 family, Hsc70, in the early events of Tau pathogenesis. We developed a model using microtubule-destabilizing compounds to

explore the consequences of loss of function for Tau and then coupled this with a toxic gain of function by promoting Tau hyperphosphorylation with GSK3β overexpression (14, 38, 39). Our results were surprising: we found that the MC1 conformation of Tau can be decoupled from Tau hyperphosphorylation. Moreover, Hsc70 facilitated MC1 formation while also enhancing Tau-mediated microtubule polymerization. Although MC1 has indeed been shown to be an early pathological structure of Tau pathology, as assessed by post-mortem tissue analysis (10) and a conformation that emerges during fibrillarization (11), our results suggest that there might be multiple phases of MC1 formation: one at the onset of Tau dysfunction, as described here, and others after Tau tangles have been seeded and become self-perpetuating (29, 40).

Based on our results, we speculate that Tau folding into the MC1 conformation after microtubule destabilization could be a protective mechanism facilitated by Hsc70 to control the disordered nature of Tau and prevent its self-assembly in the neuron. In fact, this would be expected given the known “foldase” functionality of Hsc70 and previous work showing that it prevents disordered proteins from aggregating (41,–44). However, when microtubules are destabilized in a hyperphosphorylating environment, Hsc70 still binds to Tau, but this phospho-Tau can no longer be protectively folded into the MC1

conformation. In this way, Hsc70 may be a double-edged sword; when Tau loses its function, Hsc70 can hold it in a labile folded state, returning it to the microtubule once the insult on microtubule stability has resolved. But when Tau loses its function and gains a toxic modification such as aberrant phoshorylation, Hsc70 still holds Tau but fails to protect it, perhaps inadvertently contributing to the preservation of hyperphosphorylated Tau in the cell (Figure B8). This notion that unfolded proteins are intrinsically prone to aggregation is an emerging principle for the field of protein chemistry, as recently reviewed (45).

This necessity for Tau to both lose its function and gain toxic post-translational modifications could be precisely why single mechanisms linking amyloid accumulation to Tau pathogenesis have been challenging to prove. The Tau dysfunction seen in AD might require a pleiotropic activation strategy, where a number of pathways activated by the pluripotency of amyloid must converge to promote Tau pathogenesis. In fact, amyloid is known to destabilize microtubules and activate kinases (37, 46). Thus, our model may recapitulate some aspects of amyloid-induced Tau pathogenesis and describe the very earliest part of this cascade.

Our study also shows that regardless of the neuronal environment, Hsc70 is engaged with Tau when microtubule stability is altered. Thus, inhibitors of Hsc70 ATPase activity that facilitate the release and degradation of Tau, such as those that we have recently described (14, 35), could be broadly applicable throughout the Tau pathogenic cycle. Although not investigated here, we would anticipate that Hsp90 and other chaperone proteins would also be involved in Tau processing when it disengages from the microtubule. Therefore developing diverse therapeutic strategies aimed at modulating distinct chaperone functions could be highly applicable for AD.

In summary, our data suggest that the dissociation of Tau from microtubules enhances its association with the chaperone network, specifically with Hsc70. This association, as our data suggest, seems to be crucial for Tau folding and restoration of Tau function. However, abnormal phosphorylation of Tau, coupled with its loss of function, circumvents the protective surveillance of Hsc70, and Hsc70 may actually become an unwitting accomplice to Tau pathogenesis by holding onto abnormal Tau. Based on these data, pharmacological manipulation of the Hsp70 family of proteins may prevent the disease progression in certain Tau-related neurodegenerative diseases where microtubule disruption has occurred in a hyperphosphorylating

environment, a likely scenario in AD. However, in circumstances where disease arises only from a loss of function for Tau, then inhibiting Hsc70 may accelerate pathogenicity by subverting the attempts of Hsc70 to restore Tau to the microtubule. By understanding the mechanisms involved in Tau pathogenesis, we can continue to identify therapeutic targets and strategies that may be customizable based on disease etiology.

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Abbreviations

AD: Alzheimer disease

Hsp: Heat shock protein

Hsc: Heat shock cognate

PHF: Paired helical filaments

IP: Immunoprecipitation

GSK3β: Glycogen synthase kinase 3β.

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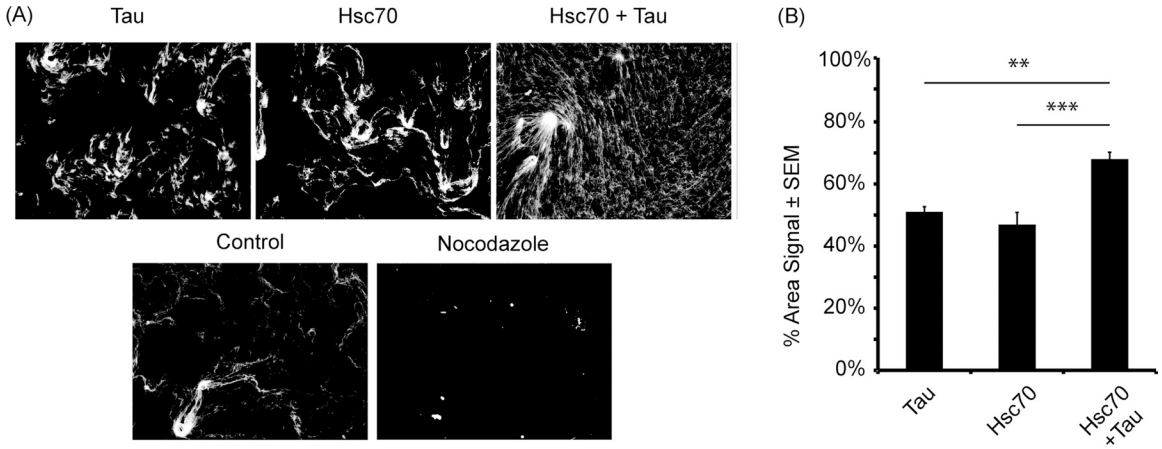
(A)
Tau
Hsc70
Hsc70 + Tau
Control
Nocodazole
(B)
100%
80%
60%
40%
20%
0%
% Area Signal ± SEM
**
***
Tau
Hsc70
Hsc70 +Tau

Figure B1

Figure B1. Hsc70 facilitates Tau-mediated microtubule stabilization. A, in vitro microtubule assembly assays were performed using Xenopus oocyte extracts supplemented with rhodamine-labeled tubulin. Recombinant proteins, Tau, Hsc70, or both in combination, were added to the Xenopus extracts, and microtubules were detected with fluorescent microscopy. Control indicates no protein added. As a negative control, microtubules were disrupted using nocodazole in the presence of Hsc70 and Tau. **B,** microtubules were quantified as the percentage of area distributed throughout the total area of the field; see details under "Materials and Methods" ($n = 19$; **, p (hsc70 versus both) < 0.001; and ***, p (Tau versus both) < 0.0001).

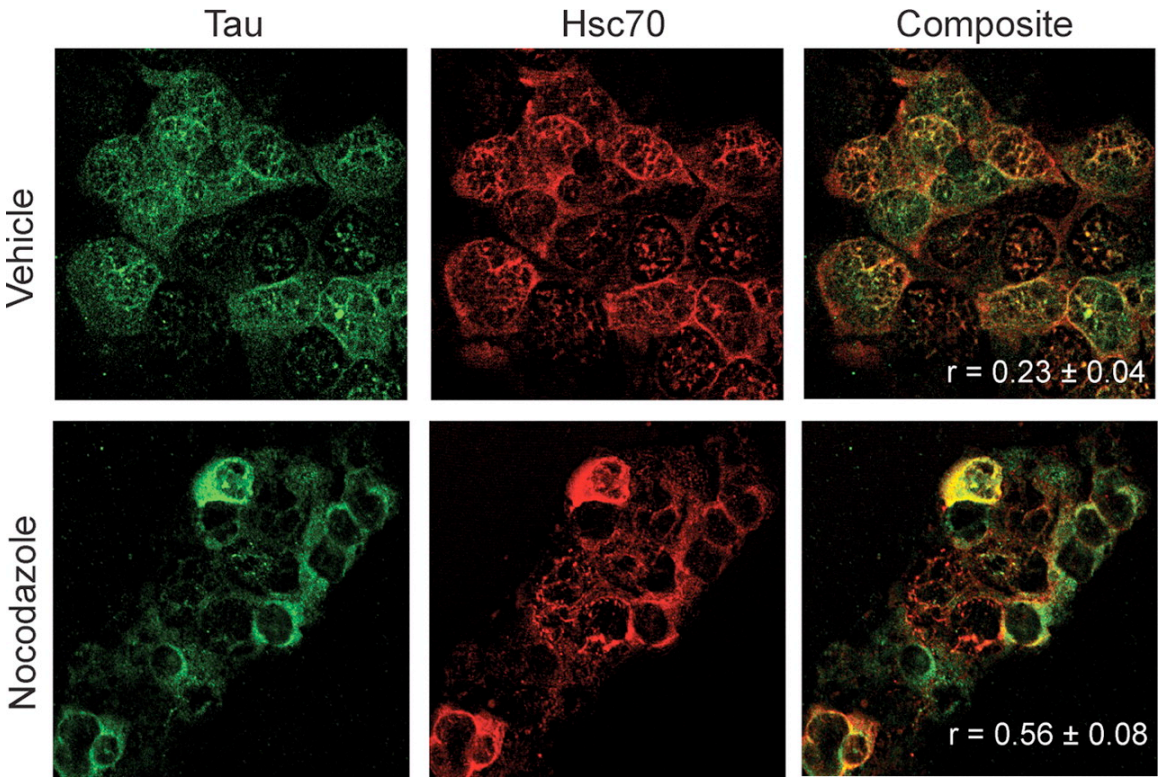

Figure B2

Figure B2. Dramatic increase in Tau colocalization with Hsc70 upon microtubule destabilization. IMR32 cells were treated with 20 μM nocodazole or vehicle control for 30 min. Immunofluorescent staining of Tau (green) and Hsc70 (red) in cells shows enhanced colocalization of these two proteins upon treatment with microtubule destabilize nocodazole.

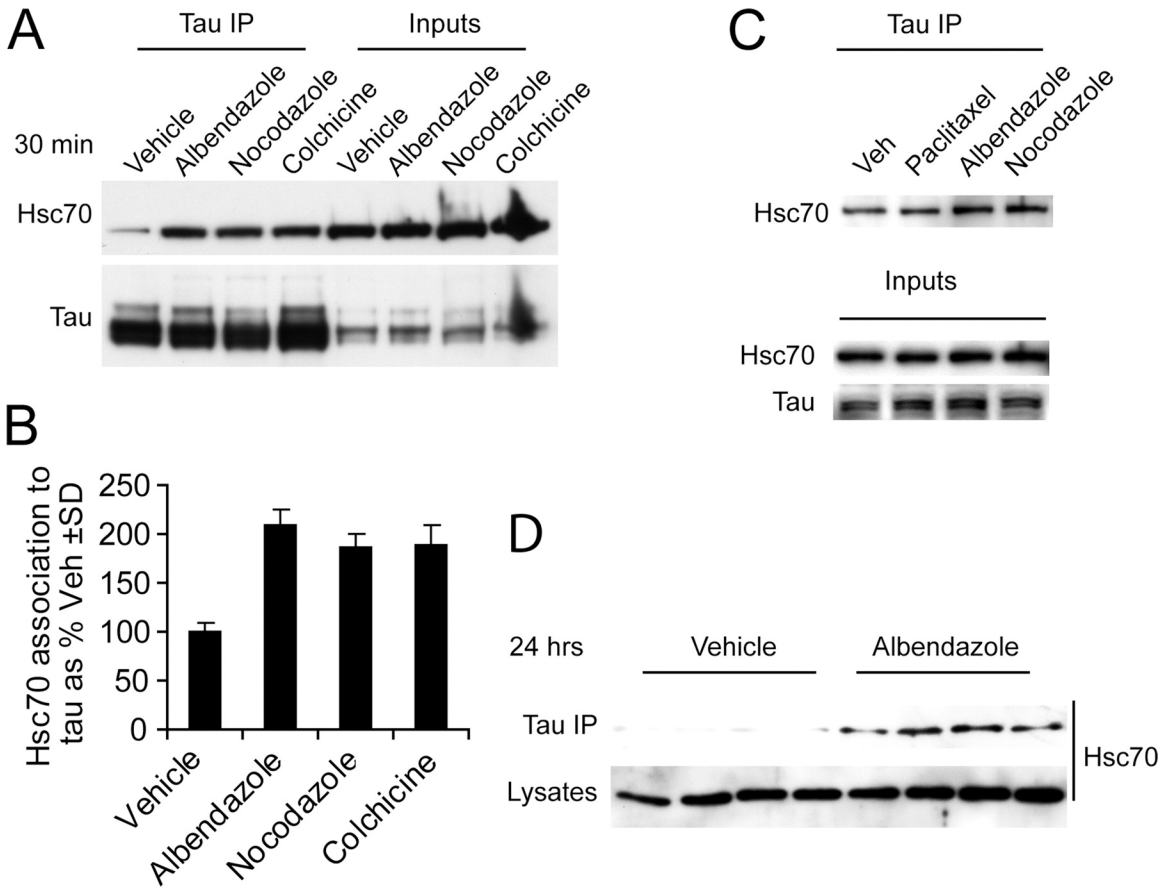
A
Tau IP
Inputs
30 min
Vehicle
Albendazole
Nocodazole
Colchicine
Vehicle
Albendazole
Nocodazole
Colchicine
Hsc70
Tau
C
Tau IP
Veh
Paclitaxel
Albendazole
Nocodazole
Hsc70
Inputs
Hsc70
Tau
B
Hsc70 association to tau as % Veh ±SD
250
200
150
100
50
0
Vehicle
Albendazole
Nocodazole
Colchicine
D
24 hrs
Vehicle
Albendazole
Tau IP
Lysates
Hsc70

Figure B3

Figure B3. In vitro and in vivo enhanced association of Hsc70 to microtubule-destabilized Tau. A, IMR32 cells treated with microtubule-destabilizing agents albendazole, nocodazole, colchicine or vehicle Me2SO as a control for 30 min. Treated cell lysates were immunoprecipitated (IP) with total Tau antibody and immunoblotted with Hsc70 antibody. **B,** quantitation of immunoblot from A after normalization to corresponding inputs shows increased binding of Hsc70 to Tau upon destabilization of microtubules by various drugs compared with vehicle control. The values are presented as Hsc70 association to Tau as a percentage of vehicle-treated cells ± S.D. **C,** In HeLa cells stably transfected with Tau were treated with microtubule stabilizer paclitaxel and destabilizer albendazole or nocodazole for 30 min. An IP with total Tau antibody followed by immunoblotting with Hsc70 antibody, showed only increased association of Hsc70 with Tau upon treatment with microtubule destabilizer not with stabilizer treatment. **D,** in vivo study was performed in mice. Brain hippocampus of mice (n = 4) were injected with albendazole or vehicle as a control. 24 h after injection, the mouse brains were homogenized. An IP with total Tau antibody followed by immunoblot analysis showed significant increased of Tau association to Hsc70 upon treatment with albendazole compared with vehicle-injected animals.

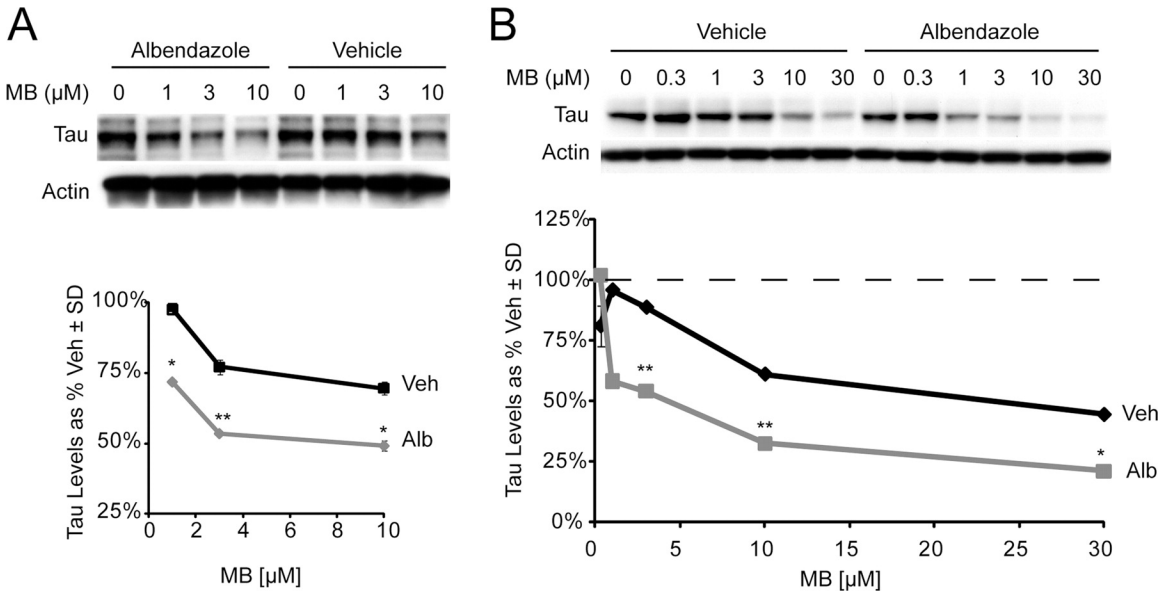
A
Albendazole
Vehicle
MB (μM) 0 1 3 10 0 1 3 10
Tau
Actin
Tau Levels as % Veh ± SD
100%
75%
50%
25%
0 2 4 6 8 10
MB [μM]
Veh
Alb
B
Vehicle
Albendazole
MB (μM) 0 0.3 1 3 10 30 0 0.3 1 3 10 30
Tau
Actin
125%
100%
75%
50%
25%
0%
0 5 10 15 20 25 30
MB [μM]
Veh
Alb

Figure B4

Figure B4. Tau released from microtubules is regulated by ATPase activity of Hsc70. A, IMR32 cells were treated with 20 μM albendazole or vehicle for 30 min, followed by 1 h of treatment with various doses of methylene blue (MB). **B,** a similar experiment as in A was performed in HeLa cells stably transfected with Tau. Quantitation plots of the Western blots after actin normalization illustrates microtubule destabilization favors further reduction of Tau by the Hsc70 ATPase inhibitor methylene blue. The gray lines depict the efficacy of the Hsp70 complex ATPase modulators alone, and black lines depict the efficacy of the ATPase modulators in conjunction with albendazole treatment. The values are presented as Tau levels as percentages of vehicle-treated cells ± S.D. *, $p < 0.05$; **, $p < 0.001$ by Student's t test.

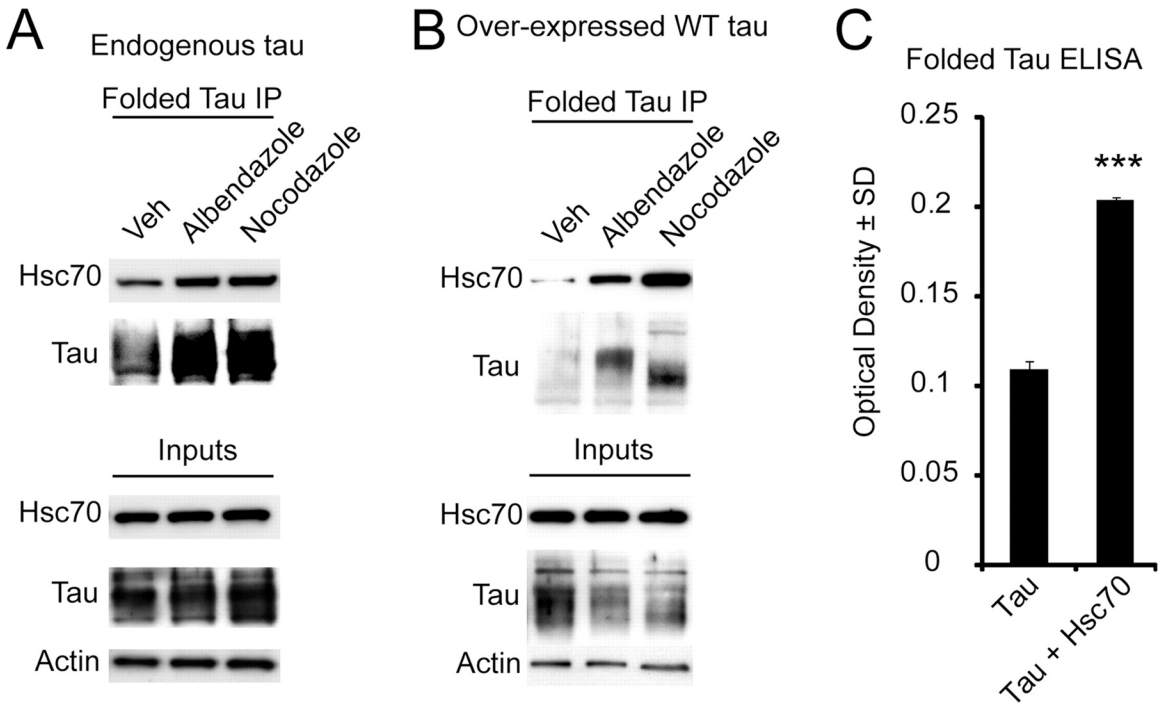
A
Endogenous tau
Folded Tau IP
Veh
Albendazole
Nocodazole
Hsc70
Tau
Inputs
Hsc70
Tau
Actin
B
Over-expressed WT tau
Folded Tau IP
Veh
Albendazole
Nocodazole
Hsc70
Tau
Inputs
Hsc70
Tau
Actin
C
Folded Tau ELISA
Optical Density ± SD
0.25
0.2
0.15
0.1
0.05
0
***
Tau
Tau + Hsc70

Figure B5

Figure B5. Hsc70 folds Tau into MC1 conformation. A, IMR32 cells having endogenous Tau were treated with 20 μM albendazole or nocodazole for 30 min prior to an IP using a MC1 (folded) Tau antibody. An increased binding of Hsc70 to folded Tau was observed upon treatment with drugs. **B,** HeLa cells stably transfected with Tau (overexpressing Tau) were treated with 20 μM albendazole or nocodazole for 30 min prior to an IP using a MC1 (folded) Tau antibody. An increased binding of Hsc70 to folded Tau was observed upon treatment with drugs. **C,** an enzyme-linked immunosorbent assay (ELISA) for folding of Tau (folded, MC1) was performed using recombinant Tau and Hsc70 in a buffer containing ATP. The plate was read at 450 nm, and the data are represented as black bars. A significant increase in Tau folding by Hsc70 suggests that Hsc70 folds Tau. The values are presented as Tau levels as percentages of vehicle (Veh)-treated cells ± S.D. ***, $p < 0.0001$ by Student's t test.

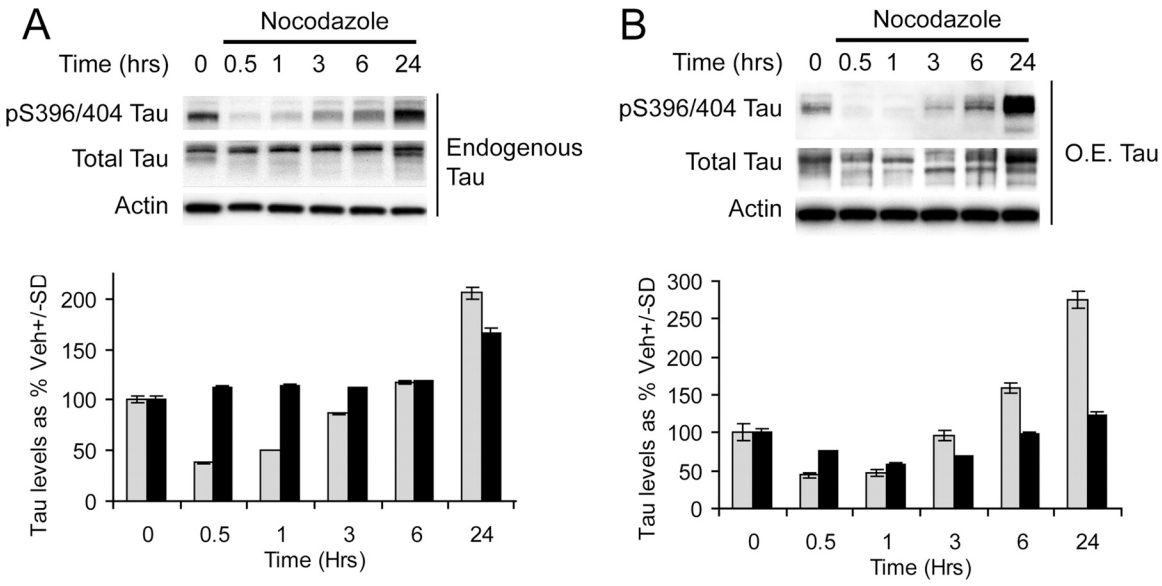
A
Nocodazole
Time (hrs) 0 0.5 1 3 6 24
pS396/404 Tau
Total Tau
Actin
Endogenous Tau
Tau levels as % Veh+/-SD
0 50 100 150 200
0 0.5 1 3 6 24
Time (Hrs)
B
Nocodazole
Time (hrs) 0 0.5 1 3 6 24
pS396/404 Tau
Total Tau
Actin
O.E. Tau
Tau levels as % Veh+/-SD
0 50 100 150 200 250 300
0 0.5 1 3 6 24
Time (Hrs)

Figure B6

Figure B6. Microtubule destabilization affects Tau phosphorylation pattern. A, IMR32 cells were treated with nocodazole for 0, 0.5, 1, 3, 6, and 24 h. Tau phosphorylation was found to be reduced initially and in later on phosphorylation was increased. **B,** HeLa cells stably transfected with Tau were treated with nocodazole for 0, 0.5, 1, 3, 6, and 24 h. Tau phosphorylation was found to be reduced initially, and later on phosphorylation was increased. Quantitation plots of the Western blots after actin normalization illustrates a time-dependent initial reduction and later on up-regulation of Tau phosphorylation. The gray bars represent phospho-Tau, and the black bars represent total Tau. The values are presented as Tau levels as percentages of vehicle-treated cells ± S.D.

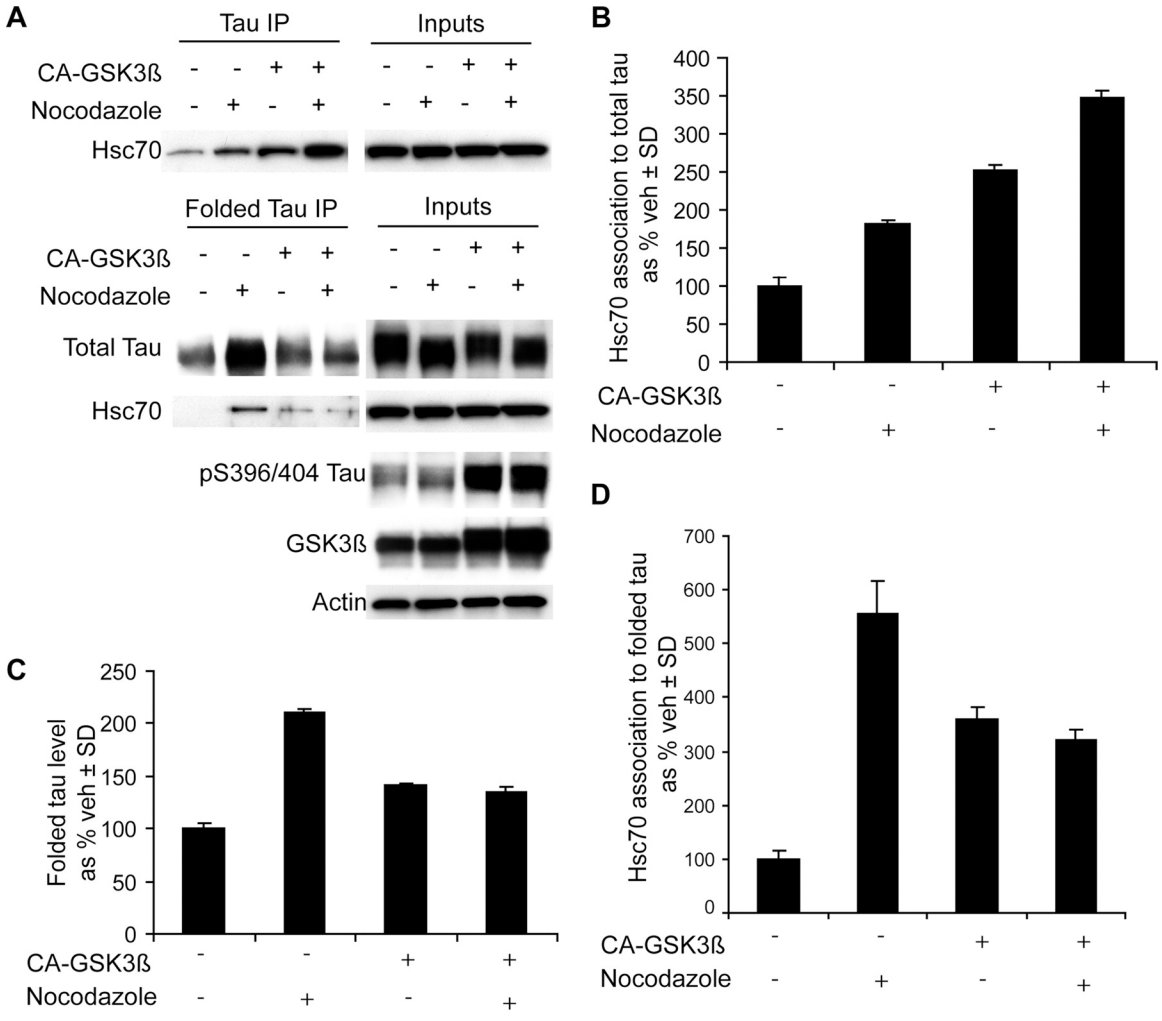
A
Tau IP
Inputs
CA-GSK3ß
Nocodazole
Hsc70
Folded Tau IP
Inputs
CA-GSK3ß
Nocodazole
Total Tau
Hsc70
pS396/404 Tau
GSK3ß
Actin
B
Hsc70 association to total tau as % veh ± SD
CA-GSK3ß
Nocodazole
C
Folded tau level as % veh ± SD
CA-GSK3ß
Nocodazole
D
Hsc70 association to folded tau as % veh ± SD
CA-GSK3ß
Nocodazole

Figure B7

Figure B7. A hyperphosphorylating environment reduces folding of Tau but enhances Hsc70 binding. A, HeLa cells stably expressing Tau were transfected with and without constitutively active GSK-3β followed by a treatment with nocodazole for 30 min. Two separate IPs were performed: one with total Tau antibody and another with MC1 antibody (folded Tau). **B–D,** quantitation of the immunoblots from A was performed after input normalization. The values are presented as percentages of vehicle (veh)-treated cells ± S.D. for Hsc70 binding to total Tau (B), total Tau following MC1 IP (C), and Hsc70 binding to MC1 Tau (D).

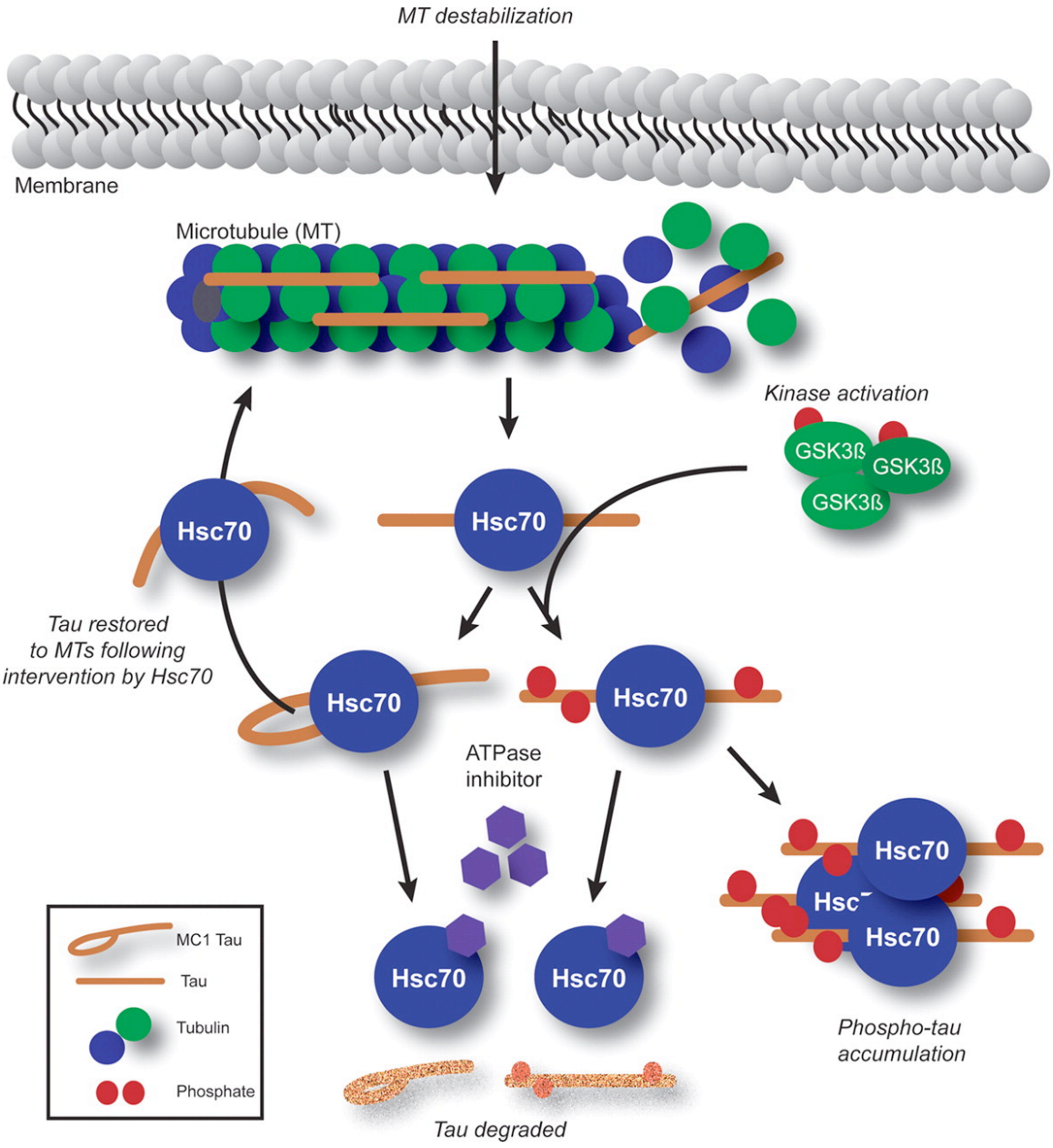
MT destabilization
Membrane
Microtubule (MT)
Kinase activation
GSK3ß
GSK3ß
GSK3ß
Hsc70
Hsc70
Hsc70
Hsc70
Hsc70
Hsc70
Hsc70
Tau restored
to MTs following
intervention by Hsc70
ATPase
inhibitor
MC1 Tau
Tau
Tubulin
Phosphate
Tau degraded
Phospho-tau
accumulation

Figure B8

Figure B8. A hyperphosphorylating environment reduces folding of Tau but enhances Hsc70 binding. A, HeLa cells stably expressing Tau were transfected with and without constitutively active GSK-3β followed by a treatment with nocodazole for 30 min. Two separate IPs were performed: one with total Tau antibody and another with MC1 antibody (folded Tau). **B–D,** quantitation of the immunoblots from A was performed after input normalization. The values are presented as percentages of vehicle (veh)-treated cells ± S.D. for Hsc70 binding to total Tau (B), total Tau following MC1 IP (C), and Hsc70 binding to MC1 Tau (D).

APPENDIX C[4]:

Neuronal Life Span Versus Health Span

Principles of Natural Selection at Work in the Degenerating Brain

Abstract

Impaired nutrient delivery to the brain due to decreased blood flow contributes to cognitive decline and dementia in Alzheimer's disease (AD). Considering this, many studies have suggested that neuroprotective agents like those used in stroke could prevent AD onset or progression by promoting cell survival. However, research in the past decade suggests that the culprit behind the cognitive loss in AD models is actually the soluble tau accumulating inside of surviving

[4]This work was previously published in the *Journal of Molecular Neuroscience* (O'Leary III, J. C., Koren III, J., & Dickey, C. A. (2011). Neuronal Life Span Versus Health Span: Principles of Natural Selection at Work in the Degenerating Brain. Journal of Molecular Neuroscience, 45(3), 467-472.), and is used here with permission of the publisher. The permissions are available in Appendix F.

neurons. In fact, tau reductions improve cognition in mouse models of AD, even those that only deposit amyloid plaques. There is emerging evidence that neuroprotection alone in these AD models may be insufficient to restore neuron function and cognition. Only when soluble tau is reduced on a neuroprotective background could memory be rescued. Thus, once a neuron begins to accumulate tau, it may survive in a malfunctioning capacity, leading to impaired electrical signaling and memory formation in the brain. These data imply that multiple drugs may be necessary to ameliorate the different disease components. In fact, strategies to preserve neurons without affecting the soluble protein burden within neurons may accelerate the disease course.

Review

Cerebral angiopathy and blood flow dysregulation in AD

Current research in Alzheimer's disease (AD) suggests that cerebral angiopathy (CA) can occur from excess neuronal secretion of amyloid beta (Aβ) and from preexisting vascular disease (Iadecola 2004; Zlokovic 2008). Aβ can be cleared from the brain by vascular transport across the blood–brain barrier. This free Aβ can bind to different transport-binding proteins like apolipoprotein J (Ghiso et al.

1993) and E (Yang et al. 1997), transthyretin (Schwarzman et al. 1994), lipoprotein receptors (Matsubara et al. 1999), and several others. Vascular smooth muscle cells (VSMC) have recently been an intense area of focus due to their ability to internalize and clear Aβ and contract the capillary (Urmoneit et al. 1997). Thus, modulation of VSMC could provide a cellular and molecular link between vascular disorders and AD. The VSMC can clear Aβ by using lipoprotein receptor-related protein-1 to sequester it (Shibata et al. 2000; Urmoneit et al. 1997). It is speculated that when the neuron begins secreting excess amyloid, the VSMC becomes saturated, allowing Aβ to accumulate. Aβ is also capable of potentiating the cell's constriction capability (Niwa et al. 2001; Paris et al. 2002). In fact, when Aβ 1–40 peptide is administered to wild-type mice by topical superfusion, the subjects display reduced resting cerebral blood flow (Niwa et al. 2001). Also, it has been found that serum response factor and myocardin are upregulated in AD tissue (Chow et al. 2007). They are transcription factor proteins that facilitate the VSMC-differentiated phenotype. Hence, AD patients may have a hypercontractile phenotype that can be further potentiated by Aβ. The amyloid cascade hypothesis is further supported by studies showing that the overexpression of amyloid precursor protein (APP) in mice is capable of causing pathologic changes before the detectable appearance of amyloid plaques. In the

PDAPP mice, dentate gyrus volume is reduced before plaque formation (Redwine et al. 2003). Mice overexpressing the Swedish APP mutation exhibit a reduction in hyperemia (Niwa et al. 2000). On the other hand, vascular disease on its own is also capable of reducing blood flow across the blood–brain barrier, creating a hypoxic state. Hypoxia has been shown to decrease the ability of VSMC to clear Aβ (Bell et al. 2009). Another complication arising from CA is that reduced blood flow leads to energy deprivation, which in turn potentiates beta-secretase 1 (BACE1) levels (O'Connor et al. 2008). Since BACE1 is the rate-limiting step of Aβ production, more BACE1 proteins create more Aβ peptide. Ultimately, a cascade can arise from CA that becomes self-perpetuating, manifesting in dementia.

Neuroprotective agents for AD

Because of the strong vascular component in AD, there has been a search for compounds that can elongate the life of neurons. Many drugs have been proposed to have possible neuroprotective effects in AD based on their ability to scavenge antioxidants and free radicals. Examples of these drugs are indole-3-propionic acid (IPA), vitamin E, and resveratrol.

IPA is an inhibitor of Aβ fibril formation, an antioxidant, and neuroprotectant. To assess its ability to protect neurons from ischemic damage, it was administered for 15 days at a 10 mg/kg dose to mice. Tissue was collected, and IPA was shown to spare neurons from ischemic damage ~300% (Hwang et al. 2009). IPA has also been shown to inhibit Aβ fibril formation, but other indole derivatives like indole 3-acetic acid, indole 3-carbinol, and tryptophol were more effective (Morshedi et al. 2007).

Vitamin E is currently in phase III of clinical trials to treat AD. However, beneficial effects of vitamin E in patients with moderate to severe AD have been modest (Brewer 2010). Vitamin E treatment of 2,000 IU slowed the functional deterioration of patients, improving their daily living, but failing to improve the mini-mental exam score (Grundman 2000; Petersen et al. 2005).

Resveratrol can promote antioxidant activity, has neuroprotective effects, and activates sirtuins and their positive effect on aging (Albani et al. 2010). This compound, derived from grapes, improves cognition and reduces plaque pathology in animal models of AD (Karuppagounder et al. 2009; Kim et al. 2007; Wang et al. 2006).

Resveratrol is one of the more promising compounds that have entered clinical trials.

Pharmacological agents like resveratrol and IPA may indeed be neuroprotective, but questions about a strategy designed to spare sick neurons are beginning to emerge. In some sense, this is in direct violation of Darwinian principles: Culling of the weak or sick from the herd is better for the species population as a whole. This same principle may be in play in the brain. Perhaps, sparing neurons that harbor aberrantly accumulated proteins could worsen brain function. Thus, extending the life of neurons that have already begun to accumulate tau may not be an effective strategy. Perhaps, the brain is able to adapt when a neuron dies, as has been shown in stroke and occlusion diseases; the plasticity in the brain can reroute processes to perform essential functions. However, when a neuron is chronically sick, but surviving, the brain may continue to route information through it, but that neuron may simply be unable to propagate the signal (Figure C1).

Pathologically visible tau or invisible soluble intermediates; what should we be targeting?

Although there are strong data demonstrating the adverse effects of vascular dysfunction and amyloid accumulation in AD, research in the last decade suggests that tau may be a better therapeutic target. Recent work has demonstrated a critical role for tau in amyloid-induced deficits of the human amyloid precursor protein (hAPP) mice (Roberson et al. 2007). Since these mice do not develop tau pathology, it was long assumed that tau did not have a role in their cognitive deficits. However, when the hAPP transgenic mice were crossed onto a tau null background, the cognitive deficits induced by amyloid accumulation were ameliorated. Moreover, tau depletion protected against both kainate- and GABAA receptor antagonist (pentylenetetrazol)-induced seizures. Furthermore, tau reduction did not change Aβ deposition, neuritic dystrophy, or aberrant sprouting. This study suggests that tau may be a better therapeutic target because it is necessary for Aβ-mediated cognitive deficits and excitotoxicity. Also, it suggests that cognition can be improved despite amyloid pathology.

Another piece of evidence that supports tau-induced cognitive decline is the creation of the rTg4510 mouse model (Santacruz et al.

2005), which is doubly transgenic for the tetracycline operator driven by the CamKII promoter and human P301L tau driven by the PrP promoter and regulated by the tetracycline-responsive element. These mice accumulate tau tangles and develop severe neurodegeneration and cognitive deficits. A large group of rTg4510 mice were trained to find the hidden escape platform in the Morris water maze (MWM) at 2.5 months of age. It was found that tau was not affecting all mice equally. As a result, the mice were split into two cohorts. To test the effect of tau reduction and pathology on cognition in a suboptimally performing cohort, tau expression was shut off at 2.5 months of age in half of the mice, while the other half received control diet. The mice were retrained and tested at 4.5 months, an age prior to onset of neurodegeneration. Suppressing tau expression for 2 months enabled mice that had previously performed poorly in the MWM to now learn and recall the location of the escape platform, while the performance of the vehicle-treated rTg4510 mice continued to decline. Next, the higher-performing cohort had doxycycline treatment initiated at 5.5 months, a point when neurodegeneration and frank tangle pathology begin in this model. These mice had improved cognitive function despite increasing tangle formation. These two studies showed that not only is soluble tau able to impair learning and memory but tangles failed to correlate with memory improvement.

A role for soluble tau in altering cognitive function was reemphasized in a subsequent study using the rTg4510 model (O'Leary et al. 2010). Seven-month-old rTg4510 mice were treated with the pleiotropic compound, methylene blue (MB), which is currently in clinical trials for AD. Direct hippocampal infusion of MB improved learning and memory in the rTg4510 mice, but neither pathology nor neuronal morphology was altered: Only soluble tau was extensively reduced.

In a more recent study, the role of caspase cleavage of tau was tested in the rTg4510 model. Caspase activation was shown to occur before tangle formation, tangle-bearing neurons were long lived, and aggregated tau was able to suppress caspase activity (de Calignon et al. 2010). Conversely, injection with a virus expressing wild-type human tau induced caspase cleavage of tau, suggesting that soluble cytosolic tau could activate caspase. This work suggests that tau tangles may protect the neuron from apoptosis, while soluble tau can induce caspase activation and possibly neurotoxicity.

There is still more evidence for the importance of soluble tau in disrupting neuronal function. Mice overexpressing wild-type human tau show no tau insolubility or neuronal loss, yet these mice have memory deficits and synaptic dysfunction. Conversely, mice overexpressing the

P301L variant of human tau did develop neuronal loss and tau insolubility, but these mice were cognitively intact and synapses were functional (Kimura et al. 2010). This concept provides further support to the notion that accumulation of soluble tau essentially clogs the neuron, but does not kill it, leading to reduced overall brain function. In fact, neuronal death and insolubility of tau are less deleterious compared to soluble tau accumulation and survival of suboptimally performing neurons.

There are several mechanisms through which soluble tau might accumulate and take on a deleterious function. Pre-fibrillar Aβ can cause microtubule disassembly, resulting in a loss of function for tau and an increased cytosolic burden of free tau (King et al. 2006; Rapoport et al. 2002). Aberrant phosphorylation and mutations associated with some tauopathies also reduce tau's affinity for the microtubules (Wagner et al. 1996), possibly enlarging the pool of tau that is not microtubule bound (Dayanandan et al. 1999). Another possible way that soluble tau becomes enriched in the cytosol would be by overexpression. Indeed, this occurs naturally in sporadic Parkinson's disease (Simon-Sanchez et al. 2009). It has been shown that overexpression of wild-type human tau leads to excess free tau in the cytosol (Andorfer et al. 2003), providing a greater opportunity for

tau to interact with itself and aggregate into a soluble nonfunctioning intermediate. Thus, perhaps when tau loses its microtubule function, it can gain a toxic function. For example, tau can mediate Aβ excitotoxicity by allowing fyn to phosphorylate the NMDA receptor subunit 2 (Ittner et al. 2010). In this study, crossing the APP23 transgenic mouse line with tau null mice disrupted the dendritic targeting of fyn. Interestingly, the same result was achieved by overexpressing a truncated form of tau that lacks the microtubule-binding domains of tau. Again, this suggests that soluble, free tau without the capacity to interact with microtubules may be even more deleterious to neuronal function.

All of these evidences strengthen the rationale for developing strategies to deplete free, soluble tau to treat AD. While reducing all tau has been thought likely to be harmful, there are several findings that suggest that the brain may be more tolerant of such a strategy than first imagined. In particular, tau knockout mice are functionally intact, due in part to compensation by MAP1a (Harada et al. 1994; Tucker et al. 2001). Moreover, fast axonal transport is not affected in tau knockout mice (Yuan et al. 2008). Considering this evidence and that many of the patients who would receive anti-tau therapies would

be elderly, reducing tau may indeed be a well-tolerated strategy to ameliorate AD symptoms and modify disease course.

Life span versus health span for neurons: do Darwinian principles apply in a degenerating brain?

The phenothiazine class of compounds includes the controversial compound, methylene blue (MB). The pleiotropy of MB has raised a number of concerns about its clinical application. More than a decade passed between the time that MB was first reported to prevent tau aggregation in solution and the time that the first reports emerged describing successful use of MB in the clinic (Wischik and Staff 2009; Wischik et al. 1996). Since then, a number of studies have begun to dissect the mechanisms contributing to the efficacy of this drug. MB was shown to reduce Aβ levels in the 3xTg-AD mouse model and improve learning and memory (Medina et al. 2011). In this study, MB treatment was given for 4 months, beginning at the age of 6 months. MB treatment ameliorated learning and memory deficits in this mouse model and reduced Aβ levels, but failed to alter tau.

Other studies have ascribed one mechanism of MB action to its ability to inhibit the molecular chaperone Hsp70. Indeed, this activity was shown to destabilize tau in cells and mice (Jinwal et al. 2009). MB treatment was recently shown to improve cognitive function in rTg4510

mice, but only when sufficiently high doses were present in the brain (O'Leary et al. 2010). Interestingly, tau tangle pathology as assessed by histology was unchanged in these mice compared to those treated with vehicle. When stereological analyses were performed on tissues from these mice, MB facilitated neuroprotection in all treated mice; however, this neuroprotection did not correlate with cognitive function. Biochemical analyses of tissue from these mice showed that soluble tau levels were reduced by MB treatment, but only in mice with high concentrations in their brain. Surprisingly, reduced soluble tau burden correlated with improved cognitive function in this study.

An additional report recently showed that reducing soluble tau burden with a purely genetic approach could improve neuronal function in the rTg4510 mice, as measured by long-term potentiation (Abisambra et al. 2010). Moreover, overexpression of wild-type human tau in mice does not beget tau insolubility or neuronal loss, but does cause cognitive dysfunction, while overexpression of mutant tau causes neuronal loss and insolubility of tau, but does not lead to memory deficits (Kimura et al. 2010). These results allow for several conclusions to be drawn. First, neuroprotection alone is insufficient to rescue memory function in tau transgenic mice, decoupling two processes that have always been intertwined. Secondly, reductions in

tau tangle pathology are not necessary for neuroprotection or cognitive improvement. Lastly, reducing soluble tau levels is necessary for cognitive improvement in this model, but it is not required for neuroprotection.

This work has several important implications regarding classical paradigms about the relationship of neuronal survival, neuronal health, and overall brain function. When soluble proteins accumulate in the cytosol, the endgame may be neuronal death, but there are likely many neurons that continue to survive for long periods of time in a suboptimal, or functionally impaired, state. This is likely due to the intracellular accumulation of "sticky" proteins. Strategies aimed at only protecting these sick neurons may ultimately be deleterious to brain function. Instead, focusing on amelioration of the protein accumulation in these neurons to restore their function may prove more therapeutic. Simply prolonging the life of nonfunctioning neurons may prevent compensatory brain plasticity mechanisms from being triggered to overcome the loss of function of a discreet circuit (Figure C1). While neuroprotective strategies may indeed be beneficial, it might be critical to couple such agents with a treatment that can reduce the soluble protein accumulation that burdens neurons in many neurodegenerative diseases. Neuroprotection has proven successful in stroke; however,

these neurons do not have toxic soluble proteins accumulating within. In neurodegenerative diseases resulting from proteotoxicity, neuroprotection without reducing the toxic protein burden may be harmful.

Conclusions

The population with AD is expected to grow tremendously within the next 20 years as the first wave of baby boomers reaches 65 years of age this year. In addition to AD, there are more than 15 other neurodegenerative diseases where tau is pathogenic. Research from the most recent decade suggests that ameliorating soluble tau protein accumulation may provide patients with greater cognitive improvement and behavioral health. This, coupled with neuroprotective agents, may be an even more effective clinical strategy. However, neuroprotection without also rescuing the soluble proteotoxic burden in neurons may be unsuccessful, essentially allowing malfunctioning neurons to evade the "survival of the fittest" principle. Thus, the brain may be unable to recognize a need to reconfigure damaged circuitry. Strategies aimed at only neuroprotection may ultimately accelerate the course of these disorders.

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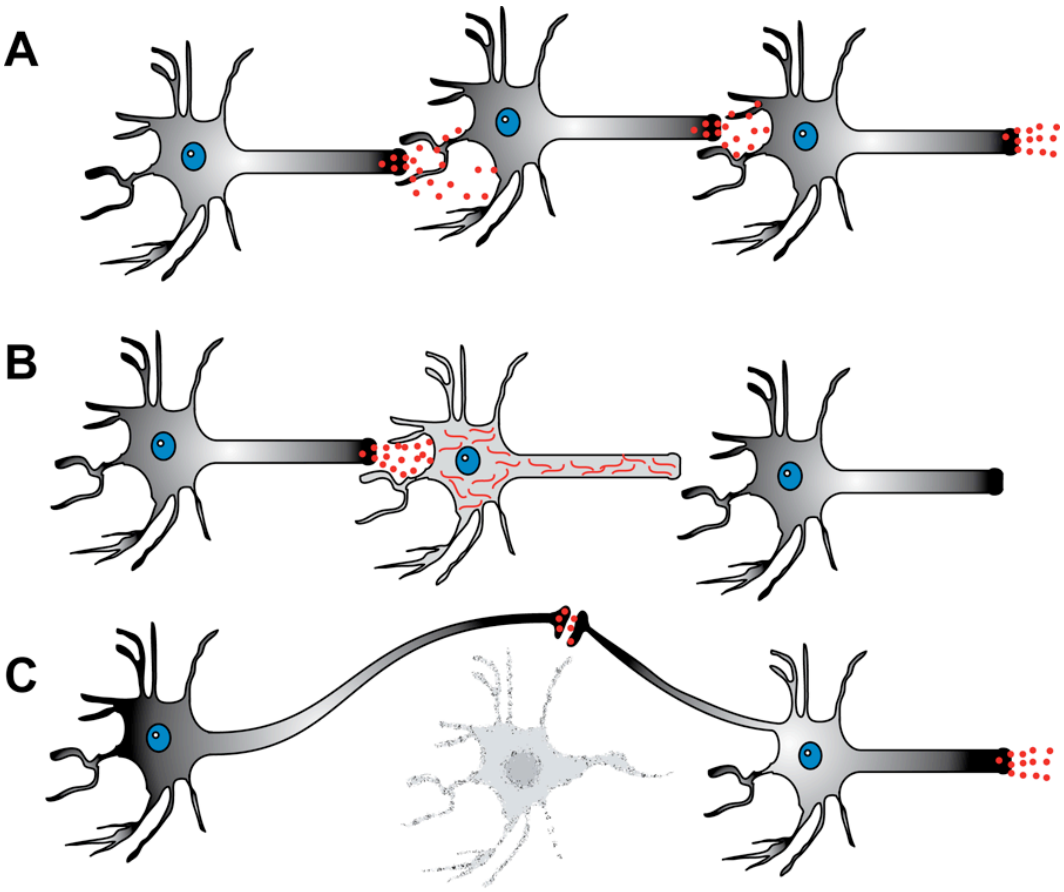

Figure C1

Figure C1. Suboptimally function- ing neurons surviving with pro- teotoxic tau accumulation subvert brain plasticity that nor- mally occurs in response to neuronal death. (A) Neuronal transmission in normal brain. (B) Suboptimally performing neu- rons due to tau accumulation cannot transmit a signal to the postsynaptic neuron, and the brain fails to reroute connectiv- ity since the neuron is not dead. (C) Neuronal death facilitates net- work rerouting, allowing the plastic brain to adapt and rees- tablish downstream connectivity.

APPENDIX D:

The Role of FKBP5 in Mood Disorders: Action of FKBP5 on Steroid Hormone Receptors Leads to Questions About its Evolutionary Importance

Abstract

Research on the FKBP5 gene and FKBP51 protein has more than doubled since the discovery that polymorphisms in this gene could alter treatment outcomes and depressive behavior in humans. This coincided with other data suggesting that the stress hormone axis contributes to the development of numerous mental illnesses. As a result, FKBP51 now lies at the heart of the research of many stress related psychiatric disorders, which has led to advances in the understanding of this protein and its role in humans and in animal models. Specifically, FKBP5-/- mice and a naturally existing overexpression of FKBP5 in 3 genera of new world monkeys have helped understand the effects of FKBP5 in vivo. This review will

highlight these finding as well as discuss the current evolutionary need for the FKBP5 gene.

Introduction

Mood disorders are characterized by feelings of sadness, frustration, loss, anger, anxiety, fear, or panic that in disease become chronic and interfere with normal life. Research in the last decade has found that dysregulation of steroid hormone receptors can cause mood disorders. The hypothalamus-pituitary-adrenal (HPA) axis, for example, which produces glucocorticoids and releases them into the blood stream, has been linked to depression (Holsboer, 2000; de Kloet et al., 2005).

Steroid hormones, produced in the periphery by endocrine glands (Belelli and Lambert, 2005), can cross the blood-brain-barrier and bind to steroid hormone receptor complexes that produce changes at the cellular level and global level, such as acting as transcription factors for gene expression upregulation, altering neuronal excitability, and modifying mood and behavior. Steroid hormone receptors are ubiquitously expressed in almost all human tissues including the brain, and are especially abundant in the parts of the brain that control mood

and emotion: the hypothalamus, hippocampus, amygdala, and prefrontal cortex (de Kloet et al., 2005). As a result, the brain is susceptible to mood disorders generated by aberrant function of steroid hormone receptors (De Kloet et al., 1998).

To this end, researchers have looked for genetic and environmental factors that increase the risk of psychiatric disease. Genome wide association studies for single nucleotide polymorphisms (SNPs) have revealed significant associations between allelic variants of the FKBP5 gene and depression (Binder et al., 2004; Tatro et al., 2010), post-traumatic stress disorder (PTSD) (Xie et al., 2010), bipolar disorder (Willour et al., 2009), peritraumatic dissociation (Koenen et al., 2005), suicide (Brent et al., 2010; Roy et al., 2010; Supriyanto et al., 2011; Roy et al., 2012), negative personality traits (Shibuya et al., 2010), and aggression (Bevilacqua et al., 2012). An environmental factor found to interact with this gene is stress (Binder, 2009; Lupien et al., 2009). The current primary biological role of FKBP51 is thought to be with the protein heterocomplex of steroid hormone receptors within the HPA axis, where it helps regulate receptor sensitivity (Jaaskelainen et al., 2011).

Due to the overwhelming evidence that stress and the FKBP5 gene are involved in psychiatric diseases, studying how this gene works is imperative to understanding the mechanisms of mood disorders and finding therapies.

Review

HPA-axis and stress

A general definition for stress is a disruption of homeostasis due to a real or perceived threat to the well-being of the organism (Chrousos and Gold, 1992; de Kloet et al., 2005). For example, upon the perception of threat, the amygdala immediately activates the autonomic nervous system and the HPA-axis.

The hypothalamic-pituitary connection provides the brain with endocrine function (Ulrich-Lai and Herman, 2009). This system evolved to allow the brain to turn the production of hormones that are made distally in the periphery off and on. These hormones travel through the blood, cross the blood brain barrier and bind to their specific receptors in distinctive brain areas.

The brain uses multiple hormones to trigger the synthesis of cortisol, which is the most abundant stress hormone. The Hypothalamic neurons synapse at the pituitary where they release corticotropin-releasing hormone (CRH), also known as corticotropin-releasing factor (CRF) (Lupien et al., 2009). The pituitary will then respond by producing adrenocorticotropic hormone (ACTH), and releasing it into the blood stream. ACTH will bind to its receptor in the adrenal gland, which is located above the kidneys, and trigger the adrenal cortex to synthesize glucocorticoids, including cortisol, and release them into the blood stream (Lupien et al., 2009).

Glucocorticoids have two main receptors in the brain, the mineralcorticoid receptor (MR) and the glucocorticoid receptor (GR). At low concentrations, the MR is the main active receptor, while at high concentrations the GR is the main active receptor since all MRs are occupied (Reul and de Kloet, 1985; Arriza et al., 1988).

The MR and GR are cytoplasmic receptors that translocate to the nucleus upon hormone binding (Datson et al., 2001). There, they activate as well as suppress transcription of many genes. The system is also self-regulating, such that activation of GR triggers a negative feedback loop that attenuates the axis and consequently stress-

hormone production (Lupien et al., 2009). Thus the rate of feedback inhibition can have dramatic impact on the length of time that the stress hormone system is active. GR is in virtually every tissue of the body, helping to coordinate the body's response to stress; however the feedback inhibition primarily occurs in the amygdala, hypothalamus, and the pituitary to shut down production. Some systemic functions of GR activation other than those affecting mood, decision-making or memory function, include increased metabolism and inhibition of inflammation (Sapolsky et al., 2000).

Cellular role and biochemistry of FKBP51 with focus on steroid hormone receptors

FKBP51 (FK506 binding protein 51) is part of the immunophilin family, a superfamily of highly conserved proteins first characterized by their ability to bind to immunosuppressant drugs (Barik, 2006). The superfamily is divided into two sequence families by the type of immunosuppressant to which it binds. FKBPs are able to bind to FK506 and rapamycin, two immunosuppressants of fungal origin. In addition to their drug-binding capability, some FK506-binding immunophilins are also protein chaperones, with the related but apparently separate ability to isomerize proline residues (Barik, 2006).

FKBP51 (FK-506 Binding Protein 51kDa, p54, FKBP54) was originally identified as a novel FK-506 binding protein capable of peptidyl-prolyl cis-trans isomerization (PPIase) activity (Wiederrecht et al., 1992). PPIases, like FKBP51, are able to change the conformation of proline residues, a unique amino acid with the capability of existing in cis and trans conformations (Barik, 2006). Proline cis-trans transitions are important for proper protein folding (Barik, 2006), yet deletion of the N-terminal PPIase domain (FK1) had little effect on FKBP51's efficacy as a chaperone. Instead, the C-terminus of FKBP51, which includes three highly degenerate 34-amino-acid repeats known as tetratricopeptide repeats (TPRs), has been found to exhibit independent protein-folding activity.

Up until recently, however, FKBP51 was relatively unstudied, particularly because its function in cellular processes was not well known. In fact, it is not understood whether PPIase activity in general is necessary for cell viability. But studies have begun to elucidate its role in the biology of the cell. For example, FKBP51 was recently found to regulate the phosphorylation of the microtubule associating protein tau (MAPT, tau), a protein whose aggregates are a hallmark of Alzheimer's disease (Jinwal et al., 2010). FKBP51, and other immunophilins, have also been found to interact with steroid hormone

receptor heterocomplexes, an interaction mediated by the chaperone heat shock protein 90 (Hsp90) (Chen et al., 1996), (Davies et al., 2005).

Three hormone receptor complexes that interact with FKBP51 are the glucocorticoid receptor (GR), progesterone receptor (PR), and androgen receptor (AR). Each, are transcriptionally active steroid hormone receptor complexes whose activation leads to a change in the transcription rate of many genes, one of which is FKBP5. Paradoxically, FKBP51 also inhibits the activation of GR (Denny et al., 2000) and PR (Hubler et al., 2003). These receptors thus have a short negative feedback loop built into their activity, while AR, on the other hand, is positively regulated by FKBP51 (Denny et al., 2005). Two questions arise from the effect of FKBP51 on GR, PR and AR. Is PPIase activity necessary? Is binding to Hsp90 necessary?

The exact mechanism of action within the steroid hormone receptor heterocomplex has been difficult to piece together, since the importance of PPIase activity of immunophilins is questionable. One study analyzing the relative importance of the PPIase and TPR domains in squirrel monkey FKBP51 rendered each of the protein domains inactive via site-directed mutagenesis and showed that while only the

TPR domain was required for FKBP51 to bind to the GR and PR complex with Hsp90, both PPIase and TPR domains were required for the FKBP51 inhibitory effect on GR to be effective (Denny et al., 2005). Other studies, however, showed that PPIase activity is not necessary for its inhibitory action on GR(Wochnik et al., 2005) but is necessary for its activating action on AR (Ni et al.). All three complexes do require Hsp90 for FKBP51 to have an effect, as Hsp90 was found necessary to be bound to FKBP51 for it to have its inhibitory action on GR (Denny et al., 2005), PR (Hubler et al., 2003), and its activating action on AR (Ni et al.). The data indicate that, perhaps due to structural reasons, the physical presence of FKBP51 may be more important than its PPIase enzymatic activity.

SNPs and FKBP51 mechanism of action

Genome-wide association studies (GWAS) have enabled scientists to look at small changes in DNA and the effects of single base pair variations called single nucleotide polymorphisms (SNPs) on gene expression and function. SNPs in FKBP5 have been associated with mood disorders and an increased risk for PTSD, suicide, and overt aggressive behavior. One particular SNP in the FKBP5 gene, rs1360780, was associated with an increased number of depressive episodes (Binder et al., 2004). A closer look at the SNP revealed three

different polymorphisms possible at the site: TT homozygotes, CT heterozygotes, and CC homozygotes. Association of these SNP variations with episodes of depression and responses to antidepressant treatment showed that individuals with TT homozygosity had more frequent episodes of depression but also a faster recovery from antidepressant therapy. SNP association studies were performed with FKBP51 and other psychiatric disorders, with findings that FKBP51 SNPs are also associated with mood disorders, many of which were formed in conjunction with childhood trauma. Increased risks of developing PTSD (Xie et al., 2010), suicide (Roy et al., 2010; Roy et al., 2012), and overt aggressive behavior were also associated with certain FKBP51 polymorphisms (Bevilacqua et al., 2012).

Mechanistically, the effect of FKBP5 polymorphisms on mood disorders is elusive. One particular area of study is the HPA axis, as FKBP51 is a known regulator of HPA-axis activity. The HPA-axis has major roles in both PTSD and depression, albeit with opposite phenotypes. Patients with Major Depressive Disorder (MDD) have a hyperactive HPA-axis, where an increased stimulation of adrenal corticotropic hormone (ACTH) and GR desensitization cause high amounts of cortisol to be released and retained in the bloodstream. PTSD patients, on the other hand, have hypersensitive GR and a

hypersuppression of cortisol (Binder, 2009). The SNP rs1360780 is thus of great interest here, as it is associated with increased risks of both PTSD and depression, despite these diseases having different GR sensitivities. For example, the TT genotype may provide mechanistic evidence on how FKBP51 could cause HPA-axis dysregulation, as it was shown that individuals with TT had higher basal levels of FKBP51 and thus very likely a greater inhibition effect on GR activity. However Depressed patients with TT genotypes had greater decreases of ACTH and cortisol levels in response to the DEX/CRH test than depressed patients with CC and TC genotypes. The study did not, however, compare the FKBP51 levels of healthy controls with the TT genotype versus depressed patients with the same genotype, and so it is unclear if FKBP51 levels are higher due to depression or if all people with the genotype exhibit increased levels of FKBP51. Nevertheless healthy individuals with the TT genotype were shown to lack a normalization of cortisol levels after stress as compared to the CC and CT genotypes; TT individuals had higher cortisol levels for a longer period of time (Ising et al., 2008). The data from the healthy controls resembled those having high FKBP51 levels, and the data of the depressed patients resembles those having low FKBP51 levels.

How do we make sense of the fact that people with the same genotype had different GR phenotypes depending on the disease state? It may be that healthy individuals only have high levels of FKBP51 in the short term, but the chronic stress state may increase FKBP51 levels in the long term, which could drive adaptive changes in GR activity. However, if FKBP51 protein levels were elevated in this genotype irrespective of disease state, it may be that another risk factor is interacting with FKBP51 to promote depression. In individuals with this genotype with probable PTSD, baseline cortisol activity was not elevated as compared to healthy controls (Binder et al., 2008), while it was in depressed patients (Binder et al., 2004). Thus it is clear that while genotypes do not consistently affect GR in the same way, the risk for mood disorders is clearly elevated by the rs1360780 SNP. This suggests that factors other than just GR must be responsible for the differential disease phenotypes.

FKBP5 animal models

Studies of FKBP51 in animal models have proven helpful in determining endogenous and stress-response levels of FKBP51 expression in different regions of the brain. A study of FKBP5 mRNA levels in the murine brain discovered that under basal conditions, the highest levels occurred in the hippocampus (Scharf et al., 2011),

especially the dentate gyrus, and the premammillary nucleus, the motor nuclei of the nervus trigeminus and the nervus facialis. Interestingly, lower levels of FKBP5 mRNA were found in the amygdala and the hypothalamic paraventricular nucleus (PVN), the latter of which is a part of the HPA axis and a stress response mediator. Stressing the mice, however, showed a marked increase in FKBP5 mRNA levels in these two regions. Both moderate and short-term stress and strong and long-lasting stress caused FKBP5 mRNA levels to rise in dose-dependent fashion. Moderate stress was caused through a 4-hour restrain, and a 1-day food deprivation paradigm was used as the long-lasting stressor. Upregulation of FKBP5 mRNA was observed in the central amygdala, the hypothalamic PVN, and the hippocampus after both conditions, with a stronger response after food deprivation in all regions. Injecting the mice with dexamethasone, a corticosteroid analog and GR agonist, also increased the levels of FKBP51 mRNA, supporting the hypothesis that the stress response and FKBP5 mRNA levels are linked to the GR.

However, while all three regions showed increased levels of FKBP5 mRNA, the relative increase differed based on the baseline levels already observed in the mouse brain. The hypothalamic PVN and the central amygdala, which had low endogenous FKBP5 mRNA levels,

had average FKBP5 mRNA increases of over 600% from baseline, while the hippocampus, which has a relatively high basal level of FKBP5 mRNA, had average increases of less than 200%. It appears that higher endogenous levels of FKBP5 mRNA caused the GR in those particular brain regions to become less responsive to corticosteroid stimulation. In cases of chronic stress, constant high levels of stress could result in potentially long-term elevated corticosteroid levels, and induce high levels of FKBP5 mRNA expression, in turn causing GR activity to become resistant.

Two additional models have been studied intensively, FKBP5-/- mice and new world monkeys. Three genera of new world monkeys have been discovered to have naturally occurring GR resistance due to excess production of FKBP51 (Scammell et al., 2001). This begs the question of whether this affects the behavior of the monkeys. An induced model of depression in monkeys exist (Clark and Gay, 1980), and extreme social stress causes coronary artery disease (Petticrew and Davey Smith, 2012), presumably due to hypercortisolemia, but it is not clear whether it is FKBP5 dependent. However, it does suggest that the hypercortisolemia seen in humans with depression could be aided by a reduction in the levels of FKBP51.

The function of the HPA axis was studied in the FKBP51-/- mice. The mice displayed a moderate GR hypersensitivity, as they produced less corticosterone (the main murine glucocorticoid) and recovered faster after dexamethasone (DEX) and stress treatment (Touma et al., 2011). The DEX/CRH test was compared between FKBP51-/- mice and healthy humans of the rs1360780 SNP. Healthy humans with the TT genotype displayed GR hyposensitivity to dexamethasone while the FKBP51-/- mice displayed GR hypersensitivity, showing that reduction in levels of FKBP51 may be a successful therapy for GR insensitivity.

In terms of protein levels and protein expression, FKBP51-/- mice exposed to chronic social defeat stress produced equal amounts of CRH mRNA as compared to wild type before and after stress, although levels did increase after stress (Hartmann et al., 2012). This demonstrated that corticosterone levels were not reduced in the FKBP51-/- mice due to the lack of HPA activation, but rather due to the FKBP51-GR negative feedback mechanism that has been characterized so well. It was found, on the other hand, that the lack of FKBP51 alters the levels of GR. In wild type mice, GR levels decreased during a 1 hr restraint stress experiment, yet in FKBP5-/- mice they increased at 15 minutes then went down slightly, but remained significantly higher than wild type. It is also possible that FKBP51 may actually be regulating the levels of GR, particularly since Hsp90 is intimately linked

to proteasomal degradation (Ballatore et al.) and Hsp90 is a part of the GR complex.

Surprisingly, general behavior was not changed in 10-16 week old (young) FKBP5-/- mice. As a result, changes in behavior after stress were studied. Young FKBP5-/- mice showed significantly less time immobile in the forced swim test than wild type mice after restraint stress (Touma et al., 2011). Moreover, FKBP51 is a protein whose expression increases with age (Jinwal et al., 2010), and young mice have low basal levels in the hypothalamus and amygdala. It may be that the young mice needed FKBP51 to be expressed after stress to modulate behavior, since basal FKBP51 levels were low. Twenty-two month old mice (old), however, displayed this phenotype in the forced swim test without prior stress (O'Leary et al., 2011). General behavior in the old mice was also unchanged, especially in learning and memory, implying that FKBP51 may have a role limited to the biology of the stress response. But this role may be protective, as stress and its related molecules are known to cause cognitive impairment (Diamond et al., 1992; de Quervain et al., 1998; Diamond et al., 1999; Diamond et al., 2004; Sandi, 2004; Diamond et al., 2005; Diamond et al., 2006).

FKBP5 and neuropsin-mediated effects in the amygdala

Recently, FKBP51 was found to be involved in stress response in the amygdala. FKBP51 was found to be upregulated by NMDA activation and caused anxiety in mice after stress (Attwood et al., 2011). This effect was dependent on the cleavage of EphB2 by neuropsin, causing EphB2 to disassociate from the NR1 subunit of the NMDA receptor and enhancing NMDA receptor current. Neuropsin is an extracellular serine protease and EphB2 is a receptor tyrosine kinase, and both are heavily expressed in the amygdala and hippocampus, with higher basal expression in the hippocampus, similar to FKBP51. After stress, neuropsin is dramatically upregulated in the amygdala. This data was not shown in the hippocampus; it would be interesting to find out if neuropsin had a similar expression profile to FKBP51 and to see the basal and stress-induced expression of FKBP51 in neuropsin knockout mice. In amygdala neuronal cell cultures, the majority of FKBP51 expression was found to be due to the activity of neuropsin and not by corticosterone, and it may be that FKBP51 has a primary role in stress behavior in a neuropsin dependent mechanism, and that its role with GR is secondary to that. Additionally, despite FKBP51 expression upregulation by NMDA receptor activation, no effects are shown in learning and memory. It may be that a large threshold of NMDA receptor activation is needed to trigger the upregulation of

FKBP51, and the large threshold would only be reached during extreme situations like stress, and thus FKBP5 would be activated to promote behavioral signatures of anxiety.

The need of the FKBP51 gene in the brain is unclear, as it seems to be extremely problematic, and its absence does not seem to have negative effects. A posited theory for the development of FKBP51 may be within the fear response. Evolutionary genetics suggest that these genes arose early in development; analysis of phylogenetic relationships among 100 FKBP domains show a clustering pattern that suggests the emergence of the FKBP genes early in eukaryotic evolution (Patterson et al., 2002), and another study suggests that FKBP proteins arose even before the divergence of prokaryotes from eukaryotes (Trandinh et al., 1992). As a result, FKBP5 may have played an important role in the evolution of the stress response. In the past we needed to exhibit a proper response to fear, since the reaction was critical to survival. The problem is, we no longer have the same life or death triggers.

Conclusion

It is clear that FKBP51 is a significant player in the human response to stress. It has a dramatic effect on the biology of steroid hormone receptors, and is upregulated in the amygdala as part of a stress response. SNPs within the FKBP5 gene are associated with mood disorders, but the mechanism of how this happens is not well understood. Surprisingly, the lack of FKBP51 does not appear to have deleterious side effects in mice and its presence causes steroid hormone receptor hypersensitivity. This not only makes FKBP51 an excellent drug target, but it also suggests that FKBP5 may be genetic baggage that no longer provides a competitive advantage for natural selection. As successful adaptation allows for a species to age, genes like FKBP5, which at one point during the evolutionary process were necessary for their survival and propagation, may actually become deleterious to the aging process. Furthermore, societal success no longer follows Darwinian principles. As a result, it may be the role of society to combat these processes; in the same way that bacteria adapt to antibiotic pressure, society may need to develop ways to suppress or even remove these genetic leftovers.

List of Abbreviations

ACTH: Adrenocorticotropic hormone

AR: Adrogen receptor

CRH/CRF: Corticotropin releasing hormone/factor

FKBP5: Gene name for FKBP51 protein

FKBP51: FK506-binding protein 51

GR: Glucocorticoid receptor

GWAS: Genome-wide association studies

HPA-axis: Hypothalamic-pituitary-adrenal-axis

MDD: Major depressive disorder

MR: Mineralocorticoid receptor

NMDA: N-methyl-D-aspartate

PPIase: Peptydil-prolyl cis-trans Isomerase activity

PR: Progesterone receptor

PTSD: Post-traumatic stress disorder

PVN: Paraventricular nucleus of the hypothalamus

TPR: Tetratricopeptide repeat

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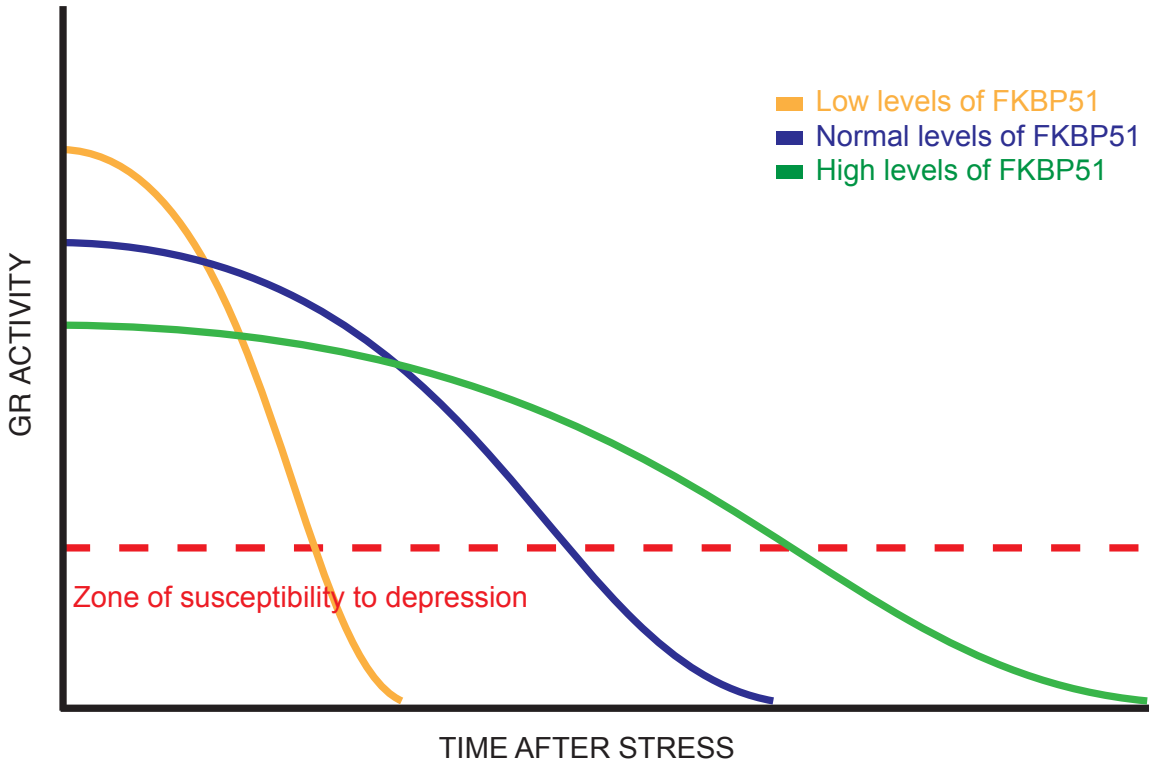
Low levels of FKBP51
Normal levels of FKBP51
High levels of FKBP51
GR ACTIVITY
Zone of susceptibility to depression
TIME AFTER STRESS

Figure D1

Figure D1. FKBP5 genotype as a modulator of GR phenotype. Everyone may be vulnerable to mood disorders involving depression following stress. However, the length of time that an individual remains vulnerable can be dramatically affected by FKBP51 levels due to its ability to decrease GR activity. FKBP51 may widen the length of time after stress that a person is susceptible to stress, while individuals with low FKBP51 levels may be in this state for a shorter period of time. While the magnitude of the acute GR response to stress following an insult may be higher in individuals with low FKBP51 levels, the length of time that an individual with high levels of FKBP51 spends in a susceptible state is longer. As a result, SNP's in the FKBP5 gene may increase the likelihood of developing psychiatric disorders by altering the levels of FKBP51. This in turn changes the responsiveness of the GR, whose balance appears to be critical for normal function. Thus, in MDD, chronic stress may have a greater effect on those individuals with too much FKBP51.

APPENDIX E:

Sequence of FKBP5 Gene

It is often that one underestimates the power of that which is small. Similarly, we commonly underestimate our potential for great achievement, precisely because we are small. Ironically, small things can have tremendous impact. Single nucleotide polymorphisms in the FKBP5 gene enlarge the size of the stress response, and interact with traumatic stress in childhood to dramatically increase the risk of major depressive disorder, and post-traumatic stress disorder. Some of these polymorphisms are also overrepresented in people who commit suicide, and develop bipolar disorder. To appreciate the size of a single nucleotide change, I have included the DNA sequence of mRNA transcript 1 from the Homo Sapiens FK506 binding protein 51. If a single nucleotide can have so much impact, maybe we can too.

Origin

GATTCGGGCC GGCTCGCGGG CGCTGCCAGT CTCGGGCGGC GGTGTCCGGC GCGCGGGCGG CCTGCTGGGC GGGCTGAAGG GTTAGCGGAG CACGGGCAAG GCGGAGAGTG ACGGAGTCGG CGAGCCCCCG CGGCGACAGG TTCTCTACTT AAAAGACAAT GACTACTGAT GAAGGTGCCA AGAACAATGA AGAAAGCCCC ACAGCCACTG TTGCTGAGCA GGGAGAGGAT ATTACCTCCA AAAAAGACAG GGGAGTATTA AAGATTGTCA AAAGAGTGGG GAATGGTGAG GAAACGCCGA TGATTGGAGA CAAAGTTTAT GTCCATTACA AAGGAAAATT GTCAAATGGA AAGAAGTTTG ATTCCAGTCA TGATAGAAAT GAACCATTTG TCTTTAGTCT TGGCAAAGGC CAAGTCATCA AGGCATGGGA CATTGGGGTG GCTACCATGA AGAAAGGAGA GATATGCCAT TTACTGTGCA AACCAGAATA TGCATATGGC TCGGCTGGCA GTCTCCCTAA AATTCCCTCG AATGCAACTC TCTTTTTTGA GATTGAGCTC CTTGATTTCA AAGGAGAGGA TTTATTTGAA GATGGAGGCA TTATCCGGAG AACCAAACGG AAAGGAGAGG GATATTCAAA TCCAAACGAA GGAGCAACAG TAGAAATCCA CCTGGAAGGC CGCTGTGGTG GAAGGATGTT TGACTGCAGA GATGTGGCAT TCACTGTGGG CGAAGGAGAA GACCACGACA TTCCAATTGG AATTGACAAA GCTCTGGAGA AAATGCAGCG GGAAGAACAA TGTATTTTAT ATCTTGGACC AAGATATGGT TTTGGAGAGG CAGGGAAGCC TAAATTTGGC ATTGAACCTA ATGCTGAGCT TATATATGAA GTTACACTTA AGAGCTTCGA AAAGGCCAAA GAATCCTGGG AGATGGATAC CAAAGAAAAA TTGGAGCAGG CTGCCATTGT

CAAAGAGAAG GGAACCGTAT ACTTCAAGGG AGGCAAATAC ATGCAGGCGG TGATTCAGTA TGGGAAGATA GTGTCCTGGT TAGAGATGGA ATATGGTTTA TCAGAAAAGG AATCGAAAGC TTCTGAATCA TTTCTCCTTG CTGCCTTTCT GAACCTGGCC ATGTGCTACC TGAAGCTTAG AGAATACACC AAAGCTGTTG AATGCTGTGA CAAGGCCCTT GGACTGGACA GTGCCAATGA GAAAGGCTTG TATAGGAGGG GTGAAGCCCA GCTGCTCATG AACGAGTTTG AGTCAGCCAA GGGTGACTTT GAGAAAGTGC TGGAAGTAAA CCCCCAGAAT AAGGCTGCAA GACTGCAGAT CTCCATGTGC CAGAAAAAGG CCAAGGAGCA CAACGAGCGG GACCGCAGGA TATACGCCAA CATGTTCAAG AAGTTTGCAG AGCAGGATGC CAAGGAAGAG GCCAATAAAG CAATGGGCAA GAAGACTTCA GAAGGGGTCA CTAATGAAAA AGGAACAGAC AGTCAAGCAA TGGAAGAAGA GAAACCTGAG GGCCACGTAT GACGCCACGC CAAGGAGGGA AGAGTCCCAG TGAACTCGGC CCCTCCTCAA TGGGCTTTCC CCCAACTCAG GACAGAACAG TGTTTAATGT AAAGTTTGTT ATAGTCTATG TGATTCTGGA AGCAAATGGC AAAACCAGTA GCTTCCCAAA AACAGCCCCC CTGCTGCTGC CCGGAGGGTT CACTGAGGGG TGGCACGGGA CCACTCCAGG TGGAACAAAC AGAAATGACT GTGGTGTGGA GGGAGTGAGC CAGCAGCTTA AGTCCAGCTC ATTTCAGTTT CTATCAACCT TCAAGTATCC AATTCAGGGT CCCTGGAGAT CATCCTAACA ATGTGGGGCT GTTAGGTTTT ACCTTTGAAC TTTCATAGCA CTGCAGAAAC CTTTAAAAAA AAAATGCTTC

ATGAATTTCT CCTTTCCTAC AGTTGGGTAG GGTAGGGGAA GGAGGATAAG CTTTTGTTTT TTAAATGACT GAAGTGCTAT AAATGTAGTC TGTTGCATTT TTAACCAACA GAACCCACAG TAGAGGGGTC TCATGTCTCC CCAGTTCCAC AGCAGTGTCA CAGACGTGAA AGCCAGAACC TCAGAGGCCA CTTGCTTGCT GACTTAGCCT CCTCCCAAAG TCCCCCTCCT CAGCCAGCCT CCTTGTGAGA GTGGCTTTCT ACCACACACA GCCTGTCCCT GGGGGAGTAA TTCTGTCATT CCTAAAACAC CCTTCAGCAA TGATAATGAG CAGATGAGAG TTTCTGGATT AGCTTTTCCT ATTTTCGATG AAGTTCTGAG ATACTGAAAT GTGAAAAGAG CAATCAGAAT TGTGCTTTTT CTCCCCTCCT CTATTCCTTT TAGGGAATAA TATTCAATAC ACAGTACTTC CTCCCAGCAT TGCTACTGCT CAGCTTCTTC TTTCATTCTA ATCCTTGCTA TTAAGAATTT AAGACTTGTG CTTACAATAT TTTTGACCTG GAGTGGATCT ATTTACATAG TCATTTAGGA TCCATGCAGC TTTTTTTGTC TTTTTAAGAT TATTGGCTCA TAAGCATATG TATACTGGTT TATGGAACTT TATTTACACT CCTCTATCAT GCAAAAAAAT TTTGACTTTT TAGTACTAAG CTTAATTTTT AAAAACAAAA TCTGTAGGGT TGACAAATAA ATAGTTGCTC TTCTACACTA GGGGTTTCAC CTGCAGGTTT GACACGCAGT TGCTCGCTTT TCCTGCCCTG TCAAGCTTCT CTGTTCTGGC GTGAGTTGTG AAAGAGTTGA AGACAGCTTC CCATGCCGGT ACACAGCCAG TAGCCTAAAT CTCCAGTACT TGAGCTGACC ATTGAACTAG GGCAAGTCTT AAATGTGTAC ATGTAGTTGA ATTTCAGTCC TTACGGGTAA ACAGATTGAG CATGGCTCTC TATTCCCTCA GCCTAAGAAA CACTCATGGG AATGCATTTG GCAACCCAAG GAACCATTTG CTTAAACCTG GAACATCTCA

CCTTTTTAAA TCCTAAAAAA CACTGGCAGT TATATTTTAA ATTAGTTTTT ATTTTTATGA TGGTTTTATC AAAAGACTTT TATTATTAGA TTGGGACCCC CTTCAAACCT AAAAATCAAG TTATTTCCTT TTATAATACT TTTCTTCCCC ATGGAACAAA TGGGATCAAT TTGTGAGTTT TTTCCTTTAA TGATAACTAA AATCCCTCTA ATTTCTCATT TATGCTTTTG TCTTTTTTAT GAAATATTTC TTTTAAAAGC CCCAGTCTCA CCTACGAAAT ATGAAGAGCA AAAGCTGATT TTGCTTACTT GCTAAACTGT TGGGAAAGCT CTGTAGAGCA TGGTTCCAGT GAGGCCAAGA TTGAAATTTG ATACTAAAAA GGCCACCTAG CTTTTTGCAG ATAACAAACA AGAAAGCTAT TCCAAGACTC AGATGATGCC AGCTGTCTCC CACGTGTGTA TTATGGTTCA CCAGGGGGAA CTGGCAAAAG TGTGTGTGGG GAGGGGAAGG GTGTGTGAGT GGTTCTGAGC AAATAACTAC AGGGTGCCCA TTACCACTCA AGAAGACACT TCACGTATTC TTGTATCAAA TTCAATAATC TTAAACAATT TGTGTAGAAG TCCACAGACA TCTTTCAACC ACCTTTTAGG CTGCATATGG ATTGCCAAGT CAGCATATGA GGAATTAAAG ACATTGTTTT TAAAAAAAAA AAATCATTTA GATGCACTTT TTTGTGTGTT CTTTAAATAA ATCCAAAAAA AATGTGACTT CCAAAAAAAA AAA

APPENDIX F:

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ABOUT THE AUTHOR

John was born in the Yucatan peninsula in Mexico to a Mexican concert pianist mother and an American photographer father. He moved to the Tampa Bay area in 1998 when he was 12 years old. Now at 28 he has developed into a musician scientist. As a musician John has had a winding path. He started piano lessons at age four and played classical piano until the age of 12. Once in the US, he picked up the tuba in the concert band and continued to develop his musicianship through high school. As a tuba player, he was selected to perform in the All-State Concert Band, and attained superior ratings in state level solo performances. As a pianist, he played in the Zephyrhills jazz band and went with the band to the State Band competition. In the 2002 Lakeside Jazz Festival John was awarded best piano soloist. John attended the University of South Florida where he studied tuba and jazz piano performance, and biochemistry. He graduated Cum Laude with a B.A. in Jazz Piano Performance, a Minor in Tuba Performance and an Honors Thesis in Biochemistry. John toured Italy and France with the USF Big Band and Jazztet combo, playing in the

2007 Umbria Jazz Festival and Juan-les-Pins Jazz Festival. John has played with jazz greats Conrad Herwig, Randy Brecker, Adam Nussbaum, LaRue Nickelson, Steve Davis, Jack Wilkins, Tom Brantley, Wycliffe Gordon, Jeff Rupert, and Marty Morell. John is a freelance musician in the Tampa Bay area and a member of the band La Lucha.

As a scientist, John completed his Ph.D. at the University of South Florida, where he was the recipient of the Ruth L. Kirschstein predoctoral fellowship from the National Institute of Neurological Disease and Stroke. John's dissertation research examines a group of proteins termed "chaperones" and their effects on the development of psychiatric diseases like Alzheimer's disease and stress-related mood and anxiety disorders, like depression. The depression component of his research was highlighted by the post-publication peer-reviewing agency Faculty of 1000, which places the article in the top 2% of all published articles in the biological and medical sciences. John has been an invited speaker at the yearly Society for Neuroscience conference in 2009, 2010 and 2012. He has also presented his research at the Midwestern Stress Response conference at Northwestern University in Chicago, IL, and the Florida chapter meeting for the American Chemical Society in Orlando, FL.